How the renin angiotensin system influences the cardiac matrix via transforming growth factor β [Beta] signaling

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How the Renin Angiotensin System Influences the Cardiac Matrix via Transforming Growth Factor β Signaling

The studies presented in this thesis were performed at the Experimental and Molecular Cardiology Laboratory, Department of Cardiology, Cardiovascular Research Institute Maastricht (CARIM).

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How the Renin Angiotensin System Influences the Cardiac Matrix via Transforming Growth Factor β Signaling

PROEFSCHRIFT

ter verkrijging van de graad van doctor aan de Universiteit Maastricht,
op gezag van de Rector Magnificus,
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volgens het besluit van het College van Decanen,
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To my parents

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1

Introduction and aims of the thesis

1. Cardiac Fibrosis and its impact on ventricular function

The normal myocardium is composed of a variety of cells: cardiac myocytes and non-myocytes including endothelial cells, vascular smooth muscle cells and fibroblasts¹. Hypertensive heart disease involves the structural remodeling of both muscular and nonmuscular compartments. It is not only the size but also the morphological and physiological quality of myocardium that accounts for pathologic hypertrophy and predisposes to ventricular dysfunction².

Remodeling implies changes that result in rearrangement of normally existing structures³. Cardiac remodeling is manifested clinically as changes in the size, shape, and function of the heart⁴. Histologically, it is characterized by a structural rearrangement of components of the normal chamber wall that involves cardiomyocyte hypertrophy, cardiac fibroblast proliferation, fibrosis, and cell death⁵. Fibrosis, a disproportionate accumulation of fibrillar collagen, is an integral feature of the remodeling characteristic of the failing heart. Accumulation of type I collagen, the main fibrillar collagen found in cardiac fibrosis, stiffens the ventricles and impedes both contraction and relaxation³. Fibrosis can also impair the electrical coupling of cardiomyocytes by separating myocytes with extracellular matrix (ECM) proteins⁵. Furthermore, fibrosis results in reduced capillary density and an increased oxygen diffusion distance that can lead to hypoxia of the myocytes⁶. Thus, fibrosis profoundly affects myocyte metabolism and performance and ultimately leads to ventricular dysfunction⁷

Fibrosis has been classified into two groups: reparative and reactive fibrosis^{8,9}. Reparative (replacement) fibrosis or scarring accompanies myocyte death. Reactive fibrosis appears as “interstitial” or “perivascular” fibrosis and is not directly linked to myocyte death. In interstitial fibrosis, fibrillar collagen appears in intermuscular spaces. Perivascular fibrosis refers to the accumulation of collagen within the adventitia of intramyocardial coronary arteries and arterioles. “Focal” and “diffuse” are also used to describe the distribution of fibrosis.

Cardiac remodeling is triggered by increased mechanical stretch; nevertheless, there are also several different factors including ischemia, hormones, and vasoactive peptides, which can modify the effects of the mechanical factor. The most common clinical situation during which remodeling is known to occur is a rather complex mixture of ischemia, stretch due to pressure overload, stress due to myocardial scar, and increased plasma levels of hormones or vasoactive peptides³.

2. Role of activated RAS in cardiac remodeling

2.1. Historical remarks

In 1889, Tigersted extracted a substance from the renal cortex, which he called “renin”¹⁰. He infused renin into the circulation, which caused elevation of blood pressure. In 1934, Harry Goldblatt demonstrated that constriction of the renal artery consistently led to hypertension^{11,12}. Additional experiments including the constriction of the aorta above and below the kidneys confirmed the presence of a renal pressor system. This discovery led to the conclusion that kidney plays a central role in blood pressure regulation. In the following years, the substance angiotensin (Ang) and its precursor angiotensinogen was discovered in plasma. In 1954, Skegg and colleagues discovered that Ang appears in two different forms, a decapeptide Ang I and an octapeptide Ang II^{13,14}. The same group also discovered that the metallopeptidase angiotensin converting enzyme (ACE) is responsible for conversion of Ang I into Ang II. These discoveries unraveled the backbone of today's well known renin-angiotensin-system.

2.2. Angitensin converting enzyme

ACE is a zinc metallopeptidase capable of cleaving numerous substrates including Ang I, bradykinin, AcSDKP and Ang (1-7)¹⁵. ACE is a tissue-based enzyme found in a number of tissues including blood vessels, heart, kidney, brain, and the adrenal gland¹⁶. Only less than 10% of ACE is found circulating in the plasma¹⁷. The importance of tissue ACE in the pathophysiology of cardiovascular disease is reflected by the findings that, despite the existence of alternative Ang II generating pathways, marked elevation of ACE occurs in almost all models of cardiac injury. After the initial observation of ACE up-regulation in pressure-overloaded, hypertrophied hearts¹⁸, marked ACE induction has been found in virtually all models of cardiac injury including volume overload¹⁹⁻²¹, myocardial infarction (MI)^{22-23,24} and heart failure^{25,26}. Additionally, increased cardiac ACE levels have been correlated with the aging process²⁷. Elevated wall stress is believed to be a critical factor for the induction of cardiac ACE because elevated enzyme levels were found exclusively in the affected ventricle²⁸.

2.3. ACE-like enzyme

In tissue, ACE is not the only enzyme capable of generating Ang II from Ang I. In the human heart, chymase is responsible for 75 % of Ang II formation²⁹. Chymase is present in granules of mast cells, endothelial cells and mesenchymal interstitial cells³⁰. Chymase levels are approximately 2-

fold higher in the ventricles compared to atria. Thus, atria exhibit proportionately higher ACE activities while the ventricles exhibited relatively more chymase levels³⁰. Recently, chymase enzyme and its activity have also been shown in dog cardiomyocytes³¹. The contribution of chymase to the local production of Ang II and its influence on cardiac remodeling has been highlighted by a recent publication, which demonstrated that chymase inhibitor was able to decrease cardiac Ang II levels and thereby inhibit collagen I and III production in failing canine heart³².

2.4. Angiotensin II

Ang II is formed by the proteolytic cleavage of C-terminal dipeptide from Ang I by both the N- and C-terminal catalytic domains of ACE with equal efficiency³³. Evidence suggests that Ang II synthesis and degradation in the heart is compartmentalized and mediated by mechanisms different from those in intravascular spaces^{29,34}. Furthermore, most components of RAS are present in the heart and are produced locally³⁵, although the extent and mechanism of local production of the RAS components and how they are produced still remains somewhat controversial^{19,36}. Regardless of the exact mechanisms, the RAS system appears to be up-regulated by mechanical stretch and other insults to the myocardium within which Ang II acts as an autocrine/paracrine factor critically affecting the progression of hypertrophy and remodeling³. Ang II has been shown to enhance protein synthesis independent of load in the intact heart as well as in the isolated myocyte^{34,37}. Ang II not only induces hypertrophy of cardiac myocytes but also hyperplasia of cardiac fibroblasts. Accordingly, the activation of the cardiac RAS and possibly, cardiac ACE, may contribute to the development of cardiac fibrosis³⁸ assuming that increased cardiac ACE would increase local Ang II. It has been demonstrated that fibrotic areas of the heart display the highest levels of cardiac ACE activity²³ and that fibroblasts themselves generate Ang II³⁹. ACE inhibitors are known to prevent the accumulation of extracellular matrix proteins and the development of fibrosis of the heart, even when pressure overload persists^{40,41}. These studies point towards the existence of an alternative blood pressure independent mechanism of ACE-induced cardiac fibrosis.

2.5. *N*-acetyl-Ser-Lys-Pro (*AcSDKP*)

AcSDKP is a tetrapeptide predominantly secreted by circulating mononuclear cells and found in various tissues including bone marrow, testes, lungs and spleen⁴². Thymosine (beta)4 has been considered as the precursor of AcSDKP^{43,44}. AcSDKP is hydrolyzed specifically by ACE and the N-terminal active site of ACE has 50-fold higher affinity for AcSDKP than the C-terminal site⁴⁵. Therefore, N-Terminal-selective ACE inhibitors may be used to selectively control AcSDKP

metabolism in target organs. ACE inhibitors, given as a single dose to normal subjects or during long-term treatment in hypertensive patients, result in plasma AcSDKP levels five- to six-fold higher and urine concentrations 40-fold higher than those of control subjects and/or patients⁴⁵. Because of its high sensitivity and specificity, the measurement of AcSDKP in plasma and urine provides a valuable tool in screening specific inhibitors of the N-terminal domain of ACE and in monitoring ACE inhibition during chronic treatment. Until recently, the only known biological function of AcSDKP was that of the inhibition of hematopoietic stem cell proliferation⁴⁶. Recent studies have demonstrated that AcSDKP does not influence blood pressure but inhibits collagen synthesis⁴⁷⁻⁴⁹. However, the exact mechanism how AcSDKP inhibits collagen deposition in the heart is not yet clear.

3. Molecular basis of cardiac fibrosis

Structural homogeneity of normal adult cardiac tissue is governed by a balanced equilibrium between stimulator and inhibitor signals of cell growth represented by peptide, steroid, or amine molecules, or a combination of these. Stimulators are normally counterbalanced by inhibitors with opposing effects on cells and matrix turnover². Loss of reciprocal regulation that normally exists between stimulator and inhibitor accounts for connective tissue remodeling. An excess of stimulators, due either to absolute stimulator overproduction or to their relative overabundance because of a deficit in inhibitor formation, can promote fibrosis.

3.1. *Lost reciprocal regulation: a paucity of local inhibitors*

The presence of myofibroblasts and their transcription and synthesis of fibrillar collagen accounts for perivascular and interstitial fibrosis. The expression of ACE is integral to the cardiac fibrosis observed after sorts of cardiac insult. As mentioned, ACE inactivates the growth inhibitors bradykinin and AcSDKP⁵⁰. AcSDKP is a growth suppressor, which is known to prevent the recruitment of pluripotent hematopoietic stem cells and normal early progenitors into the S phase of the cell-cycle by maintaining them in G₀ phase⁴⁶. Since ACE hydrolyses of AcSDKP, up-regulation of tissue ACE may degrade AcSDKP at sites of repair, thereby permitting cell proliferation. Indeed, recent publications have implicated AcSDKP as an important inhibitor of cardiac fibrosis arising from renovascular hypertension⁴⁷ and myocardial infarction⁵¹.

3.2. *Lost reciprocal regulation: overabundance of stimulators*

Humoral factors that affect the phenotype and function of cardiac fibroblasts include Ang II, basic fibroblast growth factor (bFGF/FGF-2), Transforming growth factor β (TGF β), catecholamines, and insulin-like growth factor-1 (IGF-1)^{52,53}. Among these factors, Ang II appears to be one of the most important one regulating cardiac remodeling, although other factors are also important. Ang II acts via receptors that are members of the G protein-coupled receptor superfamily⁵⁴. There are two types of Ang II receptors: type 1 (AT₁) and type 2 (AT₂). Most of the known physiological effects of Ang II are mediated through the AT₁ receptor. Binding of Ang II to the AT₁ receptor leads to activation of well-defined G protein-linked pathways, such as activation of phospholipase C (PLC), which causes the release of Ca²⁺ and subsequent activation of calmodulin kinase and protein kinase C (PKC)³. In addition to these classical pathways, AT₁ receptors can activate tyrosine kinases, such as Jak2, c-Src, and Pyk2⁵⁵⁻⁵⁷ and can transactivate cell surface receptors possessing an intrinsic tyrosine kinase domain, in particular the EGF receptor⁵⁸. Zou et al. recently reported that in cardiac fibroblasts, Ang II activated extracellular signal-regulated kinases (ERKs) through a pathway including G_i receptor coupled tyrosine kinases (eg, Src, Ras, and Raf), whereas G_q and PKC are important in cardiomyocytes⁵⁹. These differences in signal activation may lead to differential activation of genes in these two cell types.

In vitro studies of cultured cardiac fibroblasts have shown that Ang II directly stimulates fibroblast proliferation, collagen synthesis, and the expression of ECM proteins (collagen, fibronectin, and laminin) via AT₁ receptors⁵³. Ang II also regulates cardiac fibroblast function indirectly through induction of TGF β , endothelin-1 (ET-1) and osteopontin,^{53,60-62} Recent studies suggest that this indirect effect, particularly the recruitment of TGF β 1, may be the major mechanism by which Ang II controls cardiac morphology^{63,64}. This latter effect has also been directly addressed in recent studies using knockout mice. TGF β ₁-deficient mice subjected to chronic subpressor doses of Ang II showed no significant LV hypertrophy or fibrosis⁶⁵.

3.3. TGF β , *Smad* and myocardium

TGF β 1 was first characterized biochemically in 1983 based on its purification from tissue sources including human platelets and its property to induce growth of non-transformed cells in soft agar⁶⁶. Since that time, many molecules have been identified that share structural features with TGF β 1, so that the TGF β superfamily now comprises over 40 different proteins, most of which can be classified into three broad groups: TGF β s, Bone morphogenetic proteins and the activins/inhibins⁶⁷.

The first receptors belonging to this family were not cloned until almost 10 years later⁶⁸ and it is now generally accepted that the entire TGF β superfamily signals through transmembrane receptors having intrinsic serine/threonine kinase activity⁶⁹.

The signal transduction pathways for TGF β were elucidated in a burst of research work done between 1996 and 1999. The clues came initially from genetic studies of other family members in *Drosophila*⁷⁰ and in *C. elegans*⁷¹. The acronym "Smad" is a condensation of the names of the signaling molecules identified downstream of TGF β superfamily ligands in *Drosophila* (Mad)⁷⁰ and *C. elegans* (Sma)⁷¹. There are now five receptor-activated Smads and the common mediator Smad4, which directly relay the signal from the TGF β -superfamily receptor kinases to the nucleus, modulated by two inhibitory Smads⁷¹. In this canonical signaling pathway, receptor-activated Smads are phosphorylated on C-terminal serines by the type I TGF β receptor, after which they partner with Smad4. The resulting complex translocates to the nucleus where it regulates the activity of target genes, either directly by binding to cognate DNA consensus sites or indirectly by interaction with other transcription factors. Of the five receptor-activated Smads, two of them, Smad2 and Smad3, are the principal transducers of signals from TGF β receptors⁷².

TGF β participates in the regulation of cell proliferation, differentiation, apoptosis and matrix production. Moreover, TGF β plays a central role in the development of fibrosis in a variety of chronic inflammatory conditions^{73,74}. For instance, it stimulates fibroblast chemotaxis and the production of collagen and fibronectin while inhibiting collagen degradation. It can also induce expression of smooth muscle α -actin in fibroblasts and is thus considered to be one of the factors responsible for myofibroblast formation⁷⁵. In the heart, TGF β is induced by pressure overload, infarction, and Ang II infusion⁷⁶⁻⁷⁹, and its up-regulation persists for at least 4 to 8 weeks, during which concomitant expression of fibronectin and collagen is observed^{77,79}. In a more direct experimental approach, over-expression of TGF β 1 resulted in cardiac hypertrophy and fibrosis⁸⁰ while neutralization of TGF β 1 by anti-TGF β 1 antibody resulted in reduced fibrosis in pressure-overloaded rat hearts⁷⁷. Similarly, somatic TGF β 1 knockout mice failed to develop myocardial hypertrophy in response to the sub-pressure doses of Ang II⁶⁵.

Apart from Smad signaling, TGF β can activate multiple signaling pathways, including mitogen-activated protein kinase (MAPK) pathways⁸¹. Changes in Smad expression have been associated mostly with the disease conditions characterized by fibrosis. For instance, the levels of Smad2 and Smad4 are upregulated in scar tissue after myocardial infarction⁸². This effect is attenuated by the AT₁ receptor blocker losartan, suggesting the presence of crosstalk between Ang II and Smad

signaling. Conversely, Smad7, an inhibitory Smad that inhibits phosphorylation of Smads2/3, is downregulated in scar tissues after myocardial infarction⁸³.

Whereas the Smad-signaling pathway plays a prominent role in regulation of the matricellular gene transcription by TGF β , other, more multipurpose signaling pathways are also activated directly by TGF β . These include the components of MAPK pathways including Ras, Raf, ERK, JNK, and p38 as well as the regulators of cadherin junctions, RhoA and Rac⁸⁴⁻⁸⁸. Recent evidence suggests that the gene response to TGF β signaling is determined by the activation of selective signaling cascade. For example, epithelial to mesenchymal transition (EMT) in mouse mammary epithelial NMuMg cells requires p38 signaling while Smad pathway is necessary for inhibition of cell proliferation^{85,89}.

3.4. Mechanical stretch and cardiac fibrosis

Apart from humoral factors mediating cardiac fibrosis, mechanical overload may also directly contribute to cardiac matrix deposition. It has been demonstrated that cardiac hypertrophy arising as a consequence of hypertension is characterized by disproportionate increases in extracellular matrix proteins⁹⁰. The fibrosis induced by hypertension can be extensive as Querejeta and associates⁹¹ found that the collagen volume fraction of hearts from hypertensive patients was more than 2.5-fold higher than that from normal individuals. Although, increased LV mass and fibrosis seen during hypertension have been attributed to the activation of the RAS, factors other than Ang II are also important, as clearly demonstrated in recent studies in which pressure overload caused cardiac hypertrophy and fibrosis in type AT₁ receptor knockout mice⁹². Connective tissue growth factor (CTGF) is a 38Kda protein of CCN family that mediates a strong fibrotic response in different organs of the body. Apart from Ang II and TGF β 1, CTGF expression in the myocardium has been correlated with high blood pressure⁹³.

In conclusion, it is well established that activation of RAS imposes detrimental effects in cardiac structure resulting in myocardial hypertrophy and fibrosis and consequently cardiac failure. However, the exact intermediary molecular mechanisms underlying this process are not fully understood. The current thesis puts efforts to identify the crucial signaling mediators that link RAS to cardiac hypertrophy and fibrosis.

Figure 1 depicts state-of-the art knowledge at the commencement of the work for this thesis with the focused area of investigation indicated by question marks.

Aims of the thesis

The overall aim of this thesis was to investigate the mechanisms by which an activated renin angiotensin system induces cardiac hypertrophy and fibrosis and to explore novel possibilities to intervene in the underlying intracellular signaling cascades.

To investigate the signaling pathways that are activated in the heart due to increased Ang II, we utilized transgenic rats over-expressing renin and established the role of the crucial signaling mediators relevant to ventricular structure and function (chapter 3). Subsequently, we studied the effects of AcSDKP on fibroblasts proliferation, investigated the mechanisms and identified Smad signaling as the target of AcSDKP action (chapter 4). In the second stage, we studied the molecular changes and cardiac phenotype developing from exaggerated tissue ACE in transgenic rats over-expressing ACE selectively in the heart (chapter 5). Having identified TGFβ1/Smad signaling system as the crucial mediator of cardiac fibrosis, we generated cardio-selective type I TGFβ receptor deficient mice to study the role of TGFβ signaling in mediating the cardiac response to Ang II (chapter 6). Apart from neurohormonal stimuli, mechanical stretch alone is shown to initiate a cardiac matricellular response. In the final part of this thesis, we investigated the mechanisms of excess collagen production in stretched cardiac fibroblasts (chapter 7).

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2

Left Ventricular Hypertrophy: Virtuous Intentions, Malign Consequences

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Abstract

Left ventricular hypertrophy (LVH) is a key focus of current cardiovascular research with consequent evolution of novel concepts on its exceedingly complex pathophysiology. In addition to the alterations in signal transduction and disturbances in Ca^{2+} homeostasis, there are structural changes in myofilaments, disorganization of the cytoskeletal framework and increased collagen synthesis. LVH is associated with progressive left ventricular remodeling that culminates to heart failure. The modern treatment of left ventricular hypertrophy is now largely based on the hypothesis that neuroendocrine activation is important in the progression of the disease and inhibition of neurohormones is likely to have long-term benefit with regard to morbidity and mortality. Drugs specifically designed to unload the left ventricle, such as diuretics and vasodilators, appears to be less effective in reducing LV mass and improving prognosis. Thus, the evolution of treatment for LVH itself has provided much enlightenment for our understanding of the fundamental biology of the disorder.

Introduction

Left ventricular hypertrophy (LVH) is a response of the heart to a variety of biomechanical, hemodynamic and hormonal stimuli. The heart adapts to an increased demand by increasing muscle mass through the initiation of a hypertrophic response. Accordingly, myocardial hypertrophy is an early milestone during the clinical course of hypertension and heart failure and an important risk factor for subsequent cardiac morbidity and mortality¹. In most forms of cardiac hypertrophy, there is activation of quiescent signaling pathways, alteration in calcium homeostasis and an increase in the expression of extracellular matrix (ECM) encoding genes and embryonic genes.

The prevalence of cardiac hypertrophy gradually increases with age. There is a slight male preponderance reflecting the role of genetic and possibly hormonal factors². LVH remains clinically asymptomatic unless the compensatory mechanism is overrun by perpetual stress¹. Several therapeutic agents have been shown to be effective against LVH. To understand what we are up against counteracting LVH and its outcome, we will summarise some of the recent developments regarding LVH, current therapy, its targets, success and limitations. Emphasis will be given on the major pathophysiological aspects of cardiac fibrosis.

Pathogenesis

Hemodynamic stress and ischaemic events are fundamental to the development of LVH. These lead to the change in geometry, structure, and composition of the heart. Cardiac hypertrophy then constitutes a tenacious balance between adaptive characteristics and potentially deleterious structural, biochemical and molecular alterations. The adaptive mechanisms may be adequate to maintain the pump function for a short term but their capacity to sustain cardiac performance may ultimately be exceeded. It should not be surprising, therefore, that sustained cardiac hypertrophy often evolves to cardiac failure (**Figure 1**). However, it should be emphasized that not all forms of LVH progress to cardiac failure as evidenced by hypertensive rat models in which LVH is a universal phenomenon but only a proportion of those animals develop heart failure³.

The so called physiological hypertrophy that occurs during the developmental process and in elite athletes is associated with proportional increase in the length and the width of cardiomyocytes. In contrast, the assembly of contractile protein units in series characterises eccentric hypertrophy

giving rise to relatively greater increase in the length than in the width of myocytes. On the other hand new contractile protein units assembled in parallel results in a relative increase in the width of individual cardiac myocytes (concentric hypertrophy)⁴. Subsequently, LVH is accompanied by further activation of neurohormones and series of adaptive changes within the myocardium, collectively referred to as LV remodeling^{1,2}.

Fig.1. Schematic representation of the progression of LVH in course of time with concomitant remodeling process.

1. Initiation 2. Progression 3. Decompensation.
P, pressure; PV, perivascular; INT, interstitial; SERCA, sarcoplasmic reticulum calcium ATPase; BNP, brain natriuretic peptide. ↔ normal; ↑ increased; ↓ decreased.

Increased reactive oxygen species (ROS) production is recently described in relation to the pathophysiology of LVH. Vascular NADPH oxidases are implicated in the development of angiotensin II (ang II)–induced vascular smooth muscle hypertrophy but whether a similar NADPH oxidase is expressed in the heart, particularly in cardiomyocytes, and whether it plays a role in the pathophysiology of LVH are yet to be explored⁵.

Effects of neurohormones and altered genes

Neurohormones like angiotensin II (ang II), aldosterone, endothelin-1 (ET-1) and norepinephrine are frequently recruited in response to increased cardiac load. These molecules initiate cellular signals potentially toxic to the heart. Interestingly, one biological molecule activates one or more signaling pathways (pleiotropy) and inhibition of one pathway could be inefficient due to the potential recruitment of the alternative pathways. Among the neurohumoral factors, angII is most

extensively studied and is considered to be the key factor mediating cardiac hypertrophy and myocardial fibrosis. This effect of angII is presumed to be exerted via AT1 receptors⁶. However, reports from recent studies indicate that pressure overload can still produce a robust myocardial hypertrophy in transgenic mice with AT1 receptor knockout⁷. A reverse but parallel statement emerges from the study of the Zurich group, who reported a rapid reduction in myocardial mass and collagen content following valve replacement therapy in aortic stenosis patients⁸. The functional stature of neurohormones can also be influenced by genetic factors as revealed by an increased incidence of LVH in normotensive but angiotensin converting enzyme polymorphic subjects⁹. Similarly, hypertrophic cardiac myopathy has a clear genetic aetiology but not associated with co-existing cardiac or systemic disease that could provoke LVH¹⁰.

Structural and functional implications of changes in myocytes vs non-myocytes

The essential changes in cardiac myocytes during hypertrophy comprise- increased number of force-generating units eg.; sarcomeres, down regulation of sarcoplasmic Ca²⁺- ATPase (SERCA), phospholamban and upregulation of atrial and brain natriuretic peptides⁶. A decrease in α -myosin heavy chain (MHC) gene with a concomitant increase in β -MHC expression is a prominent feature in rodents but such changes are considered to contribute little on human subjects. Although these adaptive responses are considered to be beneficial, such changes, in particular calcium handling, in long run is shown to impose a defect in myocyte contractile function contributing to systolic dysfunction.

Over-expression of non-myocytic constituent proteins, in contrast, contribute largely to diastolic dysfunction. These ECM proteins are composed primarily of complex collagen weave. Given its tensile strength, collagen is a major determinant of tissue stiffness. The increase in myocardial stiffness is accompanied by a reactive activation of matrix metalloproteinases (MMP) leading to progressive degradation of ECM and thinning and dilatation of LV wall^{6,11}.

It has recently become clear that TGF- β signalling pathway plays a central role in the development of cardiac fibrosis. TGF- β is a profibrotic cytokine that orchestrates LV remodelling through the activation of Smad proteins. The activated (phosphorylated) Smads, in turn, translocate into the nucleus in order to initiate the production of ECM proteins. Studies have shown that angII increase

is related to TGF- β over expression during LV remodelling in Ren-2 rat models¹². Investigators have also demonstrated the reversal of cardiac fibrosis following the inhibition of TGF- β expression¹². The key mechanisms involved in TGF- β signalling are depicted in figure 2.

Figure 2. A simplified cartoon of TGF- β 1 signalling. TGF- β binding to a complex of type I and type II TGF- β receptors (R-I and R-II) activates receptor associated Smad proteins (R-Smad; Smad2 and 3) by phosphorylation. Phosphorylated R-Smads bind to Co-Smad (Smad4), the complexes then translocate to the nucleus and initiate transcription and translation process for extracellular matrix (ECM) protein formation.

Therapeutic considerations: finding the philosopher's stone

Treatment of LVH is aimed at the reduction of hemodynamic overload and prevention of progressive remodeling. However, reduction of cardiac after-load by vasodilators and diuretics seems to have less than adequate influence on the remission of LVH (table 1). This could probably be due to the fact that neurohumoral factors play a dominant role over hemodynamic load itself in the progression of LVH¹¹. This is supported by a *meta-analysis* of clinical trials reporting that ACE-inhibitors are most effective in reducing LV mass compared to other antihypertensive agents (table 1)¹⁰. These data are also clearly in agreement with the recently published results of LIFE study. The ability of ACE-inhibitors in preventing excess ECM production might have also played a role that favours the use of these drugs. Though impressive, but still less than optimal effects of ACE-inhibitors in the amelioration of LVH could be because of the following reasons: 1. Incomplete antagonism of renin angiotensin system 2. Existence of alternative metabolic pathway for neurohormone synthesis and 3. Inability to antagonize other biologically active systems eg. Endothelin-1, TNF and aldosterone synthesis.

Angiotensin II-receptor antagonists (AT1-blockers), candesartan was shown to cause regression of LVH in hypertensive patients. However, other reports pointed towards an increase in LV mass with losartan treatment despite its control over high blood pressure¹⁰. The effectiveness of AT1 blocker can also be influenced by their collagen lowering property. More data will be required to obtain a rational conclusion concerning the possible role of AT1- blocker in the suppression of LVH. Endothelin receptor antagonists are now shown to improve survival in LVH but further investigations are awaited before the wide-spread application of these drugs in the clinics.

Table 1. Decrease in LV mass after a mean duration of treatment of 25 weeks, with almost equi-effective antihypertensive doses of the drugs (Dahlof; American Journal of Hypertension; 1992; 5(2))

Drug	% decrease in LV mass
ACE-inhibitors	13
Ca-antagonists	9
b-blocker	6
Diuretics	7

Conclusion: LVH has currently been a subject of intensive research that has greatly deepened our insight into its pathophysiology. Treatment of LVH appears to be feasible but still not optimal. One logical approach for future LVH therapy would be to develop therapeutic strategies that more effectively antagonize the critical cellular signalling eventsAlternatively, identification of cross-talk between the key cellular signaling pathways, establishment of a single point of convergence and targeted inhibition of such factors may yield better outcomes.

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3

ERK and SMAD Signaling Both Mediate the Angiotensin II-Driven Progression Towards Overt Heart Failure in Homozygous TGR(mRen2)27

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Abstract

Background: Angiotensin (Ang) II is a key player in left ventricular (LV) remodeling and cardiac fibrosis. Its effects are thought to be transferred, at least in part, by mitogen-activated protein kinases (MAPK), but also by TGF- β 1 and the Smad pathway. In this study we sought to elucidate whether AngII related effects on LV dysfunction and fibrosis *in vivo* are mediated via MAPK or rather via Smad stimulation. *Methods:* We treated homozygous REN2 rats (7-11 weeks) with placebo, AT₁ receptor blocker (ARB) or tyrphostin A46 (TYR), an inhibitor of epidermal growth factor receptor tyrosine kinase, that blocks extracellular signal-regulated kinase (ERK) activity. *Results:* REN2 rats had LV hypertrophy (LVH) and LV dysfunction that progressed to heart failure between 10 and 13 weeks. Blood pressure normalized over time. Hormones (renin, N-ANP) and ERK were activated. P38 MAPK was not activated. Treatment with ARB prevented LVH and RVH, normalized systolic and diastolic dP/dt , N-ANP levels, and inhibited LV fibrosis (all: $P<0.05$). TYR reduced LVH, LV end-diastolic pressure, and N-ANP levels ($P<0.05$). It strongly reduced LV fibrosis ($P<0.01$). Myocardial ERK activation did not depend on AT₁ receptor signaling, as it was not affected by AT₁ receptor blockade. TYR abolished myocardial ERK activity. Smad2 activation was inhibited by AT₁ receptor blockade, however unaltered by TYR. *Conclusions:* AngII-induced LV remodeling and fibrosis are dependent on both ERK and Smad2 activation. This process is prevented by both AT₁ receptor blockade and TYR treatment, thus inhibition of either pathway is equally efficacious in restoring LV function and architecture.

Introduction

Left ventricular (LV) remodeling is characterized by LV hypertrophy and proliferation of interstitial fibroblasts, leading to cardiac fibrosis. It is clear that Ang II plays an eminent role in LV remodeling by its ability to recruit various growth factor pathways, such as mitogen-activated protein kinases (MAPKs)¹⁻⁵, and TGF- β 1⁶. Recent *in-vivo* studies confirmed MAPK activation in different models of LV hypertrophy⁷⁻¹¹. In addition, we have recently shown that TGF- β 1 is crucially involved in the development of cardiac fibrosis in AngII driven LV hypertrophy⁶. The effects of TGF- β 1 are largely conveyed by Smad2, which is phosphorylated upon TGF β stimulation¹².

However, it remains unclear whether the cardiac changes caused by AngII are governed by one dominant growth factor system, or whether different systems convey different aspects of LV hypertrophy. To investigate which growth factor system is crucial in the development of AngII-driven cardiac hypertrophy, we employed the homozygous rather than the heterozygous TGR(mRen2)27 rat model, since we noted in our previous study⁶ that this variant, when left untreated, develops an accelerated heart failure phenotype, and survives until the age of 11-12 weeks. This accelerated progression from compensated LV hypertrophy to the full syndrome of LV failure makes it an ideal and rapid model to study AngII-dependent progression from LV hypertrophy towards overt failure.

Given recent findings that the adverse effects of AngII are importantly conveyed by the epidermal growth factor receptor (EGF-R) activation of extracellular signal-regulated kinase (ERK), both in vascular smooth muscle cells¹³, fibroblasts¹⁴, and LV hypertrophy *in-vivo*¹³, we hypothesized that inhibition of ERK activity would be more effective than inhibition of p38 MAPK in preventing AngII-driven LV remodeling. Therefore, we compared the effects of either an inhibitor of EGF-R tyrosine kinase (Tyrphostin) or a specific p38 MAPK-inhibitor (SB 239063) in homozygous TGR(mRen2)27 *in-vivo*. We compared this to treatment with a low-dose (subpressor) of the AngII type 1 (AT₁) receptor blocker candesartan, since we had shown earlier that subpressor AT₁ receptor blockade can prevent the transition towards maladaptive hypertrophy in this model. We determined whether these interventions attenuated LV remodeling, and whether this is associated with alterations in ERK, p38 MAPK, and Smad2 signaling.

Methods

Animals

We employed male TGR(mRen2)²⁷ (REN2) rats, that were *homozygous* for the expression of the mouse renin (Ren)-2 transgene¹⁵. Transgene status was confirmed with Southern Blotting. Transgene-negative Sprague Dawley (SD) rats from the same breeding colony (Max Delbrück Center for Molecular Medicine, Berlin-Buch, Germany) were used as controls. Animals were fed ad libitum, with free access to tap water, and housed according to institution rules (in a single room of the animal facility with 12:12 hours light-dark cycles, and ventilation, temperature (22°C), and humidity control (50%) present). All animal experiments were approved by an animal studies review committee, and in accordance with institutional guidelines (University of Groningen, The Netherlands).

Design of the study

First, since the *homozygous* REN2 rats have not been systematically described, we studied the time-course of LV remodeling to accelerated heart failure in these homozygous REN2 rats, by determining various parameters at 6, 10, and 13 weeks. We studied LV hypertrophy (LVH), LV function, and neurohormonal activation, and compared them to control Sprague Dawley (SD) rats (group size was $n = 8$ in each group). Based on the data obtained in this study, we chose the time-frame for the subsequent treatment study. In this latter study, we evaluated 7-week old controls (SD plac; $n=7$), and REN2 rats that were treated during four weeks with placebo (REN2 plac; $n=6$), or with the AT₁ receptor blocker candesartan (REN2 ARB; $n=8$), the EGF-R blocker-TYR, (REN2 TYR; $n=6$), or the specific p38 MAPK inhibitor SB239063 (REN2 p38-I; $n=7$). To control the effect of treatment, we also treated SD rats with the ARB, TYR, and the p38-I.

Drugs and minipumps

The following drugs were used: a specific AT₁ receptor blocker (candesartan, a gift from ASTRAZENECA, Zoetermeer, The Netherlands) that was dissolved in 0.1 M NaHCO₃ in saline (0.9% NaCl), according to the manufacturer's guidelines. Final dose administered was 0.05 mg/kg/day; this dose was chosen not to affect the development of high blood pressure, comparable to the low dose ARB we used previously in heterozygous TGR(mRen2)²⁷. Second, an inhibitor of epidermal growth factor receptor (EGF-R) tyrosine kinase TYR (Sanverttech, Heerhugowaard, The Netherlands), that was dissolved in 0.01% DMSO and H₂O, according to manufacturers guidelines.

Final dose administered was 600 µg/kg/day, based on a previous study¹⁶. The p38 MAPK-specific inhibitor SB239063 (a generous gift from Dr. Robert N. Willette, Department of Cardiovascular Pharmacology, GlaxoSmithKline, King of Prussia, PA, USA), was dissolved in 0.05% DMSO and 25% PEG400, and administered in a dose of 15 mg/kg/day.

All drugs were administered to the rats by an osmotic minipump (Alzet osmotic minipumps, Palo Alto, CA, USA; types 2004 for the ARB and 2ML4 for TYR and p38-I). These minipumps will release their content at a constant rate for at least 30 days. Minipumps were aseptically filled by injection, and weighed before and after filling. We calculated the volume weight injected to control for adequate filling of the pumps. Rats were anaesthetized with 2-4% isoflurane in a gas mixture of N₂O/O₂ (2/1). Minipumps filled with the ARB and the p38-I were inserted subcutaneously, and minipumps filled with TYR were placed intraperitoneally. After sacrifice, the minipumps were collected and weighed to determine the dispensed volume.

Determination of LV function

Anaesthesia was induced as described above. The right carotid artery was cannulated with a pressure transducer catheter (Micro-Tip 3French, Millar Instruments Inc., Houston, TX, USA) connected to a 486 PC equipped with an analog-to-digital converter and appropriate software (Millar Instruments, Germany). The catheter was advanced into the aorta and the LV. The zero-pressure baseline was obtained by placing the pressure sensor in 38°C saline before measurements. After a 3-minute period of stabilization, maximal LV pressure (LVP), LV end-diastolic pressure (LVEDP), and heart rate were recorded. Hereafter, the catheter was withdrawn to measure systolic blood pressure in the aortic root. As indices of global contractility and relaxation, we determined the maximal rates of increase and decrease in LVP (systolic dP/dt and diastolic dP/dt), corrected for peak systolic LVP.

Plasma N-terminal ANP and renin levels

Blood obtained from the aorta was anti-coagulated with EDTA and immediately centrifuged (4°C, 1500g for 15 minutes). Plasma was stored at -80°C until assayed. Plasma N-terminal atrial natriuretic peptide (N-ANP) was measured with a commercially available radioimmunoassay kit (Biotop, Oulu, Finland). Active plasma renin concentration (APRC) was measured by determining the amount of AngI generated from angiotensinogen with radioimmunoassay. Both assays were previously described¹⁷.

Tissue procurement and immunohistochemistry

After hemodynamic measurements, the heart was excised and arrested in diastole by immersing it in ice-cold KCl (2M). The LV and right ventricle (RV) were dissected free and weighed, and two LV midpapillary slices were cut out. One LV slice was prepared for immunohistochemistry by fixing in 4% paraformaldehyde in phosphate-buffered saline (PBS, pH=7.4) for 12 hours, and paraffin embedding. A midventricular section was snap-frozen in liquid nitrogen at -80°C. To stain microvascular endothelium, we employed the lectin Griffonia Simplicifolia I (GSI; Sigma Chemical Co, The Netherlands), as described earlier¹⁸. Sections (3 µm) were dewaxed and dehydrated, and endogenous peroxidase was inhibited by methanol/H₂O₂ (0.3%) for 15 min. The sections were incubated overnight with the biotinylated lectin GSI (1:100) at room temperature, followed by incubation with the alkaline phosphatase conjugated biotin-avidin complex (1:200) for 30 min at room temperature and development with Fast Blue BB⁺ (Sigma). Collagen was stained with Sirius-Red, as described¹⁸. To stain phosphorylated p38 (p-p38), sections were pre-treated with 0.1% protease at 37°C for 30 minutes, and endogenous peroxidase was inhibited by methanol/H₂O₂ (0.3%) for 15 min. Sections were incubated with a polyclonal anti-rabbit p-p38 MAPK (1:50; New England Biolabs, Westburg BV, Leusden, The Netherlands) for 60 minutes. Detection was performed with a secondary antibody (goat anti-rabbit, peroxidase-conjugated (GARpo, 1:50; DAKO A/S, Glostrup, Denmark), a rabbit anti-goat tertiary antibody (1:50; DAKO), and subsequent treatment with diaminobenzidin for 10 min. To stain phosphorylated ERK (p-p44/42), sections were pre-treated with 0.1% protease (37°C, 30 min), and endogenous peroxidase was inhibited by methanol/H₂O₂ (0.3%) for 15 min. Antigen retrieval was employed by incubating sections in 1 mM EDTA (pH 8.0) in a microwave (700 Watt, 8 min). Sections were incubated with a monoclonal anti-mouse p-p44/42 (1:50, DAKO). Detection was performed using peroxidase-conjugated secondary rabbit anti-mouse antibody (1:50, DAKO), subsequent GARpo antibody (1:50, DAKO), and treatment with diaminobenzidin for 10 min. All reactions were carried out at room temperature. Sections were counterstained with hematoxylin.

Morphometry

Microvessel density was calculated as number of microvessels/mm². For quantifying fibrosis, computerized planimetry was performed in 6-10 randomly selected fields per section, in 5 to 8 rats per group, in a random manner. Perimysial and endomysial collagens were included, but perivascular and epimysial collagens were excluded. Collagen volume fraction (CVF) was

calculated as the sum of all areas stained positive for Sirius-Red divided by the sum of all myocardial areas in each rat. Microvessel density was measured by visualizing seven to ten microscopic high power fields (X400) from each rat, with transversely sectioned myocytes (the total area ranging from 257802 μm^2 to 368288 μm^2), and digitally recorded with appropriate software (Image Analysis, Leica, Germany). The microcirculation was defined as the system of vasculature that is supplying the tissue between an arteriole and a venule¹⁸.

Protein isolation and western blotting of P38, ERK, Smad2 and collagen

We described this previously^{20,12}. In short, whole-cell protein extracts were obtained from LV tissue specimens after homogenization (using rotor/stator device) in cold RIPA buffer (Phosphate Buffered Saline (PBS) with 0.1% SDS, 1%NP40, 0.5%sodium deoxycholate, 1mM sodium orthovanadate, 4 μM leupeptin, 1 μM pepstatin A, and 0.3 μM aprotinin). Samples were centrifuged (15,000 g, 20 min, 4°C) to remove unbroken cells and the resulting supernatant was further subjected to centrifugation (15,000 g, 20 min, 4°C). Total protein concentration was measured in the supernatant using the bicinchoninic acid method, as described²¹. Prestained low-molecular-weight marker (Bio-Rad, Hercules, CA, USA) and 30 μg of protein from samples were separated on 10% SDS gels by SDS-PAGE. Separated protein was transferred onto 0.45 μM polyvinylidene difluoride (PVDF) membranes that were blocked at room temperature for 1 h in Tris-buffered saline with 0.2% Tween 20 (TBS-T) containing 5% skim milk and probed with primary antibodies. Primary antibodies (Cell Signaling Technologies, Westburg BV, Leusden, The Netherlands) against total and phosphorylated ("P"-) ERK, P38, and Smad2 were all diluted 1:1000 in TBS-T. (The P-Smad2 antibody was a generous gift from Dr. Peter ten Dijk, Netherlands Cancer Institute, Amsterdam, The Netherlands). Collagen I (1: 3000) and III (1: 500) were purchased from Abcam, the Netherlands. Secondary antibodies (horseradish peroxidase (HRP)-labeled anti-rabbit IgG, Cell Signaling Technology) were diluted 1:4000 in TBS-T. Bands on Western blots were visualized by enhanced chemiluminescence (ECL) or ECL+Plus (Amersham, Arlington Heights, IL, USA), according to manufacturer's instructions.

Statistical analyses

Data are given as means $\pm$ SEM. In the time-course study, SD rats were compared to REN2 rats at the 3 different time-points using Student's *t*-tests. In the treatment study, untreated SD rats were compared to untreated REN2 rats using Student's *t*-test. The three treatment REN2 groups were

compared using ANOVA) with a Dunnett *post-hoc* analysis to correct for multiple comparison, using untreated REN2 as a control category. Analyses were performed using the statistical package SPSS 10.0 (Chicago, IL, USA). *P*-values of <0.05 were considered statistically significant.

Results

Time-course of LV function, hormones, MAP kinase activation

Blood pressure (Figure 1) was elevated after 6 and 10 weeks ($P<0.05$ vs SD), but decreased after 13 weeks ($P<0.05$ vs SD). LV and RV were hypertrophied at all time-points ($P<0.01$ vs SD). LV function (table 1) gradually deteriorated over time, and after 13 weeks, rats were in overt heart failure with hypotension, severely disturbed hemodynamics, pleural effusion, and symptoms of dyspnea and lethargy. Plasma N-ANP was increased already at early time-points, and increased further at later time-points (Figure 2). Total ERK was comparable between the groups but phosphorylated ERK was significantly increased at 6 and 10 weeks (Figure 3). Total and phosphorylated p38 MAPK were comparable between REN2 and SD rats at all time-points (not shown).

Figure 1. Graph showing the time-course of systolic blood pressure in homozygous REN2 and SD rats. Solid circles (●) represent data from SD rats and non-filled circles (○) are for REN2 rats, * $P<0.05$ vs. SD.

Figure 2

Figure 3

Figure 2. Bar diagram showing the time-course of neurohormonal activation. Solid circles (●) represent data from SD rats and non-filled circles (○) are for REN2 rats. A, plasma N-ANP levels, B, plasma renin levels, * $P < 0.05$ vs. SD.

Figure 3. Western blots and densitometric analyses showing total and p-ERK 1/2 and p38 levels in SD and REN2 rats at 6 and 10 weeks. Left panel: total and p-ERK 1/2 at 6 and 10 weeks, right panel: total and p-p38 MAPK, * $P < 0.05$ vs. SD.

Table 1. LV and RV weights and LV function in REN2 rats and SD controls in different age of life

	6 weeks		10 weeks		13 weeks	
	SD	REN2	SD	REN2	SD	REN2
LVW	2.8±0.0	4.0±0.1*	2.2±0.0	3.7±0.1*	2.3±0.2	3.8±0.1*
RVW	0.8±0.0	0.9±0.0*	0.6±0.0	1.0±0.1*	0.7±0.0	0.9±0.1*
Systolic dP/dt	67±3	54±5	91±3	70±1*	84±3	65±4*
diastolic dP/dt	-69±3	-52±5*	-90±3	-66±1*	-80±3	-61±5*
LVEDP	3±1	6±1	12±2	19±1*	11±1	17±2*

Systolic and diastolic dP/dt (mmHg/sec) are corrected for LV peak systolic pressure. LVW and RVW: left and right ventricular weight corrected for body weight. LVEDP: LV end-diastolic pressure. *: $P < 0.05$ vs. age-matched SD.

Table 2. LV and RV weights, LV function, and neurohormones of placebo-treated SD and placebo, ARB and tyrphostin treated REN2 rats.

	SD	REN2	REN2	REN2
	placebo	placebo	ARB	TYR
LVW	2.3±0.0	4.2±0.1*	2.2±0.0#	3.6±0.2#
RVW	0.7±0.0	1.1±0.2*	0.6±0.0#	0.8±0.1
SBP	109±2	101±9	95±4	115±1
systolic dP/dt	94±1	78±2*	98±2#	83±2
diastolic dP/dt	-93±2	-71±3*	-85±3#	-75±2
LVEDP	7±1	14±2*	12±1	7±2#
N-ANP	1.7±0.2	4.8±0.9*	2.1±0.3#	2.7±0.3#
Renin	11.6±4.0	42.2±8.4*	75.9±11.0#	44.7±8.5

Abbreviations: see table 1. SBP: systolic blood pressure. ARB: angiotensin receptor blockade. TYR: Tyrphostin A46. *: $P < 0.05$ REN2 placebo vs. SD placebo. #: $P < 0.05$ REN2 treatment groups vs. REN2 placebo.

LV function and hormonal activation after treatment

Systolic blood pressure was similar in all groups, indicating that the low dose ARB or the other form of treatment did not affect the dynamics of blood pressure in this model. LV function was clearly impaired in untreated REN2 rats compared to SD (Table 2). ARB prevented LV dysfunction by restoring systolic and diastolic dP/dt , and reduced LV and RV hypertrophy. Moreover, it normalized plasma N-ANP levels, whereas plasma renin levels were increased by ARB. TYR only partially restored LV hypertrophy, but completely normalized LVEDP, and also clearly decreased plasma N-ANP. Although p38 inhibition attenuated LV hypertrophy, it failed to improve hemodynamic parameters (not shown). We treated 3 additional SD groups with an ARB, TYR, and p38 MAPK-inhibitor. These treatments did not yield any effect in the SD rats (data not shown).

MAPK and Smad2 activation after treatment

Phospho-p38 MAPK was not different in SD or REN2 rats, and none of the treatments affected p38 MAPK activation (not shown). ERK was activated in untreated REN2 rats compared to SD rats (Figure 4C), and TYR blocked this activation (Figure 4C). Paradoxically, treatment with an ARB caused an increase in LV ERK activity. Smad2 was activated in REN2 rats compared to placebo (Figure 4D). Levels of Smad7 was not detectable by western blotting in any experimental group (not shown). Treatment with ARB reduced Smad2 activity, whereas neither TYR nor p38 MAPK inhibition affected Smad2 phosphorylation (data not shown).

Morphometric analyses

Both collagen I and III were markedly increased in the untreated REN2 rats (Figure 5). Treatment with both the ARB and tyrphostin decreased myocardial collagen deposition. P38 MAPK inhibition did not affect these parameters (not shown). Microvessel density was decreased in REN2 rats. This was amenable to ARB and TYR (data not shown).

Immunohistochemical staining for phosphorylated ERK

Phosphorylated (activated) ERK was found in the non-myocytic compartment of the heart. Focal staining of endothelial cells was observed, and intense staining was found in media of arterioles (Figure 6A, 6B). Furthermore, spindle-shaped interstitial cells, either fibroblasts or pericytes, were focally positive (6C, 6D, 6E). No staining was observed in myocytes. P38 MAPK was not phosphorylated (activated) in heart sections of any of the REN2 rats, irrespective of the treatment (data not shown).

Figure 4. Levels of p-ERK 1/2, and p-Smad2 normalized to total ERK1/2 and Smad, respectively in candesartan, tyrphostin and placebo-treated REN2 rats compared to SD controls. A, levels of p-ERK 1/2 and a representative blot showing increased ERK activity in candesartan and placebo-treated REN2 rats, B, Western blots and densitometric analyses of p-Smad2, showing increased Smad2 activity in tyrphostin and placebo-treated REN2 rats, **P*<0.05 vs. SD placebo, #*P*<0.05 vs REN2 placebo, PC: positive control.

Discussion

The main findings of this study are two-fold. First, we demonstrate for the first time that the homozygous *REN2* rats, when left untreated, progresses very rapidly (within 4-6 weeks) from hypertensive cardiac hypertrophy towards hypotensive heart failure. Secondly we demonstrate that this transition is transduced importantly via activation of both ERK and Smad2. Both of these pathways seem pivotal to the development of this phenotype.

The homozygous TGR(mRen2)27 as an accelerated model for AngII-driven heart failure
We document for the first time that the *homozygous* variant of the transgenic hypertensive TGR(mRen2)27 displays a rapid progression from cardiac hypertrophy towards overt heart failure that closely resembles the course of events in human heart failure, yet in a ‘fast forward’ development. This provides a unique model that closely mimics the pathophysiology of

hypertensive heart failure yet within a considerable shorter time frame than other models, especially spontaneously hypertensive rats (SHR)⁷⁻⁹. This progression towards cardiac failure is characterized by severe LV dysfunction, progressive increase in plasma N-ANP, interstitial fibrosis, a relative lack of capillarization, and activation of two important growth factor cascades: ERK (p42/44 MAPK) and Smad2, the major signaling molecule of TGF- β 1. Until now, only 2 reports described the phenotype of the homozygous REN2 rat, in contrast to the heterozygous REN2 rat, which has been studied extensively. Lee and colleagues¹⁵ described excessive systolic hypertension, severe end-organ damage and a mortality of 50% after 15 weeks.

Figure 5. Myocardial collagens content in REN2 and SD rats. A, densitometric analysis of collagen I. B, densitometric analysis of collagen III. Both collagen I and collagen III were increased in the hearts of REN2 rats that was reduced by treatment with both candesartan and tyrphostin. Plac, placebo, Tyr, tyrphostin, ARB, AT₁ receptor blocker, * $P < 0.05$ vs. SD placebo, #, vs. REN2 placebo.

We confirmed the mortality rate, showed that TGF- β 1 is critically involved in this model, and were able to prevent excess mortality by TGF- β 1 blockade⁶. In the present study, homozygous TGR(mRen2)²⁷ were initially hypertensive but at later time points blood pressure was decreased. Since the decrease in blood pressure was accompanied by a further increase in plasma N-ANP, the decrease in blood pressure may indicate the progressive loss of LV function.

AT₁ receptor blockade and TYR

Although both AT₁ receptor blockade and TYR similarly prevented structural and functional deterioration, we postulate that these beneficial effects are conferred via different pathways. TYR inhibited ERK phosphorylation, and completely prevented the development of adverse changes.

These effects seem specific for ERK, since p38 MAPK inhibition failed to do so. AT₁ receptor blockade on the other hand, in a dose that did not attenuate blood pressure, also prevented the development of phenotype. It did, however, not inhibit ERK activity, but rather Smad2 phosphorylation, which was unaltered by TYR.

Figure 6. Immunohistochemistry for p-ERK1/2. A, p-ERK 1/2 was present in endothelial cells of capillaries (arrows). B, marked signal seen in the media of arterioles, and also in the endothelial cell layer (arrows). C, staining seen in interstitial cells, not in myocytes. D and E, tangential sections reveals activated ERK 1/2 in spindle-shaped cells, Original magnification X400.

Therefore, the pathophysiology of the transition from LV hypertrophy towards LV failure seems to be based on two important mechanisms. Firstly, activation of EGF-R kinase and its downstream effector ERK42/44 is possibly regulated by blood pressure since ERK was not inhibited by low dose (non-hypotensive) ARB. Second, the activation of Smad2 might be dependent on direct signaling through AT₁ receptor. It is interesting to see that the inhibition of only one of the two pathways suffices to completely block the development of the phenotype.

Comparison of MAPK activation to other models

Various experimental models have yielded contradictory data on the activation patterns of the various MAPKs. Behr and colleagues reported activation of p38 MAPK in stroke-prone SHR receiving a high-salt/high-fat diet⁹, and they could prevent the end-organ damage with p38 MAPK inhibition. Rats with Dahl-salt sensitive hypertension show p38 and JNK MAPK activation during the hypertrophic stage, but this signal progressively diminishes, so that the signal is even less during heart failure than in controls⁷. ERK is not activated in this model. Haq and colleagues showed no activation of p38, ERK, and JNK in *human* LVH during the compensated, hypertrophic stage. During heart failure however, all 3 MAPKs were activated²³. In line with this, Flesch et al. found a de-activation of ERK and JNK upon mechanical unloading, whereas p38 became activated²⁰. Although MAPK activation patterns of different models and species are hard to compare, it seems that ERK activation like we report in the TGR(mRen2)27 resembles what is found in human heart failure. Various mouse models have been generated where ERK and p38 MAPK have been activated. In contrast to our findings, these models suggested that ERK might be related to compensatory hypertrophy²⁴. Here, we report a (small) reduction of LVH by TYR. TYR also lowered plasma N-ANP, which is compatible with the finding of Aoki et al, who showed that AngII is able to induce ANP secretion by myocytes via ERK activation²⁵. It is however very likely that the differences in phenotype can be simply explained by the fact that in the transgenic mice, activation was continuous and confined to myocytes, while in our model activation of ERK was seen only in non-myocytes. Immunohistochemical localization of activated ERK revealed specific signals in the non-myocytic compartment, i.e. fibroblasts and endothelial cells. These findings are compatible with the distinct anti-fibrotic effect and a stimulatory effect of microvessel density of TYR, which we momentarily describe. Results from *in-vitro* studies support these findings. Sano et al. found that both p38 and ERK are specifically upregulated in cardiac fibroblasts upon AngII stimulation²⁶. This effect is conferred via the AT₁, not the AT₂ receptor²⁷. *In-vitro*, inhibition of the ERK-pathway inhibits collagen synthesis by fibroblasts²⁸. Li, Chen, and Mehta found that in endothelial cells

AngII-induced endoglin expression (a proliferative and pro-angiogenic factor) is mediated by ERKs, and that this could be abolished by ERK blockade²⁷. Similarly, endothelial cell remodeling by AngII was reported to be ERK-mediated²⁹. Fischer and colleagues found that angiotensin II induced ERK in endothelial cells, but not in myocytes⁵. Thus, different MAPKs may be activated in different cell compartments. The findings that TYR was especially efficacious in inhibiting fibrosis and loss of microvessels is compatible with the described ERK activation in these cell types.

AT₁ receptor blockade inhibits Smad2 phosphorylation

One remarkable finding is that, although treatment with the AT₁ receptor blocker candesartan yielded positive effects on all outcome parameters, ERK activity was increased rather than decreased. This may implicate that AT₁ receptor blockade exerts its effects via other pathways than by interfering with ERK-activation alone. We have previously shown that TGF- β 1 inhibition with tranilast inhibits fibrosis, LV remodeling, and mortality in this model⁶. TGF- β 1 confers its effects largely via the Smad pathway³⁰. We have recently shown that the proliferative effects of TGF β -1 in fibroblasts are transduced via Smad2 phosphorylation¹². Thus, we explored the level of Smad2 activity, and found that Smad2 activity is increased in REN2 rats, and decreased by ARB, whereas it remains stable during TYR treatment. This suggests that also *in-vivo*, AngII confers its pro-fibrotic effects, to an important extent, via Smad2 phosphorylation. Others also suggested that, in contrast to the mitogenic effect of ERK, the co-mitogenic effect of AngII is mediated independently of ERK and p38 MAPK³¹, e.g. via TGF- β 1. Tharaux et al.³² showed that AngII-induced fibrosis could be prevented by TGF- β 1 scavenging, and ERK activation is involved in this, although ERK inhibition alone could not abolish AngII-induced fibrosis. Blanchette et al.³³ showed that TGF- β 1 signaling increases ERK activation, suggesting that this pathway may be a distinctively different target for inhibition. ERK-mediated phosphorylation of Smads in response to TGF- β 1 is essential in maintaining TGF- β -dependent transcriptional activation^{34,35}.

Thus, we postulate that AngII and high blood pressure both recruit ERK activation, and that AngII also induces TGF- β 1 signaling. Furthermore, we suggest that the combined activation of both ERK and Smad is necessary for the progression towards heart failure.

Study limitations

The fact that we used an EGF-R tyrosine kinase inhibitor-TYR, to inhibit ERK activation represents a limitation of our study. Although EGF-R is directly upstream of ERK in the MAPK cascade, the effects of TYR may potentially be ascribed to the inhibition of these upstream components. It has, however, been established in various cell types, like cardiac fibroblasts¹⁴ and epithelial cells¹⁶, that ERK activation is directly modulated by inhibition and activation of EGF-R.

Perspectives

We describe that the homozygous REN2 rat, a model of AngII-driven LV dysfunction fibrosis and heart failure, exhibits an increased activity of both ERK and Smad2. Blockade of both the AT₁ receptor and ERK improved LV performance and architecture. However, ERK activity was decreased only by TYR treatment and was not affected by AT₁ receptor blockade. Smad2 activity, which is increased in the REN2 rat, is inhibited by AT₁ receptor blockade, but not by TYR. Thus the transition from compensated LVH towards heart failure needs both ERK and SMAD signaling. As a result, to prevent the transition towards heart failure, traditional AngII receptor blockade still seems at least as efficient as isolated blockade of down-stream signaling cascades.

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Schematic presentation of the main findings

4

N-acetyl-Seryl-Lysyl-Aspartyl-Proline inhibits phosphorylation of Smad2 in cardiac fibroblasts

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Abstract

N-Acetyl-Ser-Asp-Lys-Pro (AcSDKP) is a specific substrate for the N-terminal site of angiotensin converting enzyme (ACE) and increases five-fold during ACE inhibitor therapy. It is known to inhibit the proliferation of hematopoietic stem cells and has also recently been reported to inhibit the growth of cardiac fibroblasts. We investigated its mode of action in cardiac fibroblasts by assessing its influence on transforming growth factor beta1 (TGF β 1) mediated Smad signaling.

AcSDKP inhibited the proliferation of isolated cardiac fibroblasts ($p < 0.05$) but significantly stimulated the proliferation of vascular smooth muscle cells. Flow cytometry of rat cardiac fibroblasts treated with AcSDKP showed significant inhibition of the progression of cells from G0/G1 phase to S phase of the cell cycle. In cardiac fibroblasts transfected with a Smad sensitive luciferase reporter construct, AcSDKP decreased luciferase activity by $55 \pm 9.7\%$ ($p = 0.01$). Moreover, phosphorylation as well as nuclear translocation of Smad2 was decreased in cardiac fibroblasts treated with AcSDKP. To conclude, AcSDKP inhibits the growth of cardiac fibroblasts and also inhibits TGF β 1 stimulated phosphorylation of Smad2. Since AcSDKP increases substantially during ACE inhibitor therapy, this suggests a novel pathway independent of angiotensin II by which ACE inhibitors can inhibit cardiac fibrosis.

Introduction

Although some attempts have been made to understand the biological behaviour of cardiac fibroblasts, far more studies have been done on cardiomyocytes. On the other hand, fibroblasts are the cells that comprise more than 2/3rd of the total myocardial cell volume¹. It has been shown that stimulation of (myo)fibroblast activity leads to the excessive myocardial fibrosis which is implicated in the decompensation of left ventricular (LV) function^{2,3}. Different growth factors play an important role in myocardial fibrosis of which TGFβ1 is a crucial cytokine involved^{4,5}. TGFβ signals via plasma membrane serine/threonine kinase receptors followed by the activation of cytoplasmic effectors like Smad proteins. Phosphorylation of Smad2 and its subsequent translocation to the nucleus are the critical steps in cell signaling through this pathway. Inhibitory Smads, Smad6 and 7 antagonize these processes^{6,7}. The effects of TGFβ1 on growth of cell depend on the types of cell subjected to it. Accordingly, TGFβ1 stimulates the growth of fibroblasts⁸ but on the other hand is a potent inhibitor of vascular smooth muscle cell growth⁹. Therefore, any factor that inhibits TGFβ1 signaling would be expected to have opposite effects depending on the type of cell studied.

Upregulation of ACE has been suggested to induce cardiac fibrosis by increasing the formation of angiotensin II (AngII) and ACE inhibitors effectively inhibit this response. However, ACE-inhibitors have been shown to reduce cardiac fibrosis even when the plasma levels of Ang II were not reduced indicating that Ang II is not the only factor that relates ACE to cardiac fibrosis¹⁰. ACE can metabolize a number of substrates, resulting into either activation or inactivation of their function. AcSDKP is one of the most recently identified peptides which is inactivated by ACE¹¹; however, the role of AcSDKP in the cardiovascular system was not clearly established until recently.

AcSDKP is an endogenous tetrapeptide secreted by bone marrow and is found in plasma^{12,13}. AcSDKP is degraded specifically by ACE both *in vitro* and *in vivo*, and its plasma level rises substantially during ACE inhibitor therapy¹⁴⁻¹⁶. It is known to regulate the proliferation of hematopoietic progenitors and¹⁶ and is also shown to reduce the proliferation of hepatocytes as well as renal fibroblasts^{17,18}. These studies indicate that an increase in AcSDKP may inhibit the growth of different types of cells. Recent studies have shown that AcSDKP attenuates the synthesis of DNA and collagen by cardiac fibroblasts both *in vivo* and *in vitro*^{19,20}. Considering the pivotal

role of TGF β 1 in cardiac fibrosis, we hypothesized that AcSDKP may interfere with TGF β 1 signaling, and thereby exert anti-fibrotic effects. Therefore we examined whether AcSDKP modulates the TGF β 1-induced signaling pathway in cardiac fibroblasts.

Materials and Methods

Reagents

AcSDKP was purchased from Bachem (Bubendorf, Switzerland). Lisinopril was a generous gift from Merck, USA. Dulbecco's Modified Eagle Medium (DMEM), foetal bovine serum (FBS), L-glutamate, penicillin and streptomycin were purchased from Gibco, BRL. Culture plates were obtained from Costar, the Netherlands. StreptABC complex/HRP- kit (ABC) and 3,3'-diaminobenzidine tetrahydrochloride (DAB) were purchased from DAKO, Denmark.

Cell Culture

The rat aortic smooth muscle cell line (PAC1 cell) was a kind gift from Dr. G. van Eys, Department of Molecular Genetics, University of Maastricht. Cardiac fibroblasts were isolated from 2- days old neonatal Sprague Dawley (SD) rats. Cultured neonatal rat ventricular nonmyocytes have been shown to be predominantly fibroblasts²¹ and were prepared as described previously²². All experiments were performed on cells from the second passage. Each preparation contained >95% fibroblasts as determined by their appearance under phase contrast microscopy and immunocytochemical staining with an antibody against cardiac troponin-C and His-52.

Both cardiac fibroblasts and PAC1 cells were cultured in DMEM supplemented with 10% and 5% FBS respectively along with 1% L-Glutamate and 50 units/ml Penicillin and 0.1g/L Streptomycin and were incubated at 37°C in a humidified 5%CO₂ atmosphere. Lisinopril (100 nmol/l) was added in the medium to prevent degradation of AcSDKP by ACE present in the serum. All cells were placed in low-serum medium (0.1% FBS) for 24 hours before the experiment. This protocol was approved by an animal studies review committee, and in accordance with the institutional guidelines (University of Groningen, The Netherlands).

CellTitre 96® Aqueous One Solution Cell Proliferation Assay

CellTitre 96® aqueous one solution cell proliferation assay (Promega) is a colorimetric method used to determine the number of viable cells in culture. This reagent contains a novel tetrazolium

compound, MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium, inner salt; MTS and an electron coupling reagent (phenazine ethosulphate; PES) which is bio-reduced by viable cells into a colored formazan product that is soluble in tissue culture medium. The conversion of MTS into colored formazan product is presumed to be accomplished by NADH or NADPH present in the viable cells.

Cell Cycle Analysis: Fluorescent activated cell sorter (FACS) analysis was performed according to Nicoletti et al²³. Cellular uptake of propidium iodide was measured by FACS calibre cytofluorometer. Flow cytometry was performed on 5000 cells/sample using an EPICS Elite flow cytometer (Coulter, Miami, FL) equipped with a single 488-nm argon laser. Data were analysed by using Modfit LT software program (Verity, inc.). Data were expressed as the percentage of total cells counted.

Coulter Counter Assay

PAC1 cells from each experimental group were subjected to count in a coulter counter. Counting was done per 2000 µl of cell solution at 100 times dilution. Since these experiments were performed in two different laboratories growth was assessed differently for cardiac fibroblasts and PAC1 cells.

Luciferase Assay of TGFβ1-Inducible Smad Binding Element (SBE) Promoter Activity

Neonatal rat cardiac fibroblasts were plated at a density of 12000 cells/well in 24 well clusters. Reporter plasmid pGL3ti(SBE)4 was a generous gift from Dr. L.J.C. Jonk, Department of Genetics, University of Groningen, Haren, The Netherlands. This plasmid contains 4 copies of Smad binding element (SBE) driving the firefly luciferase reporter gene. SBE is composed of the sequence CAGACA in the promoter of the *junB* gene which is induced by TGFβ1²⁴. Renilla luciferase reporter plasmid pRL-CMV was purchased from Promega which was used for normalization of transfection efficiency. Cells were transfected with Saint (Saint, Groningen, The Netherlands) according to the manufacturer's protocol, lysed with passive lyses buffer (Promega) and luciferase activity was measured by using the Dual Luciferase Reporter Assay System (Promega). The efficiency of transfection reaction was established by comparing the luciferase activity of transfected cells with a dilution series of purified luciferase enzyme.

Smad Protein Determination

Different proteins were measured by Western blotting with the following antibodies- Smad2 and Smad7 (goat polyclonal IgG; Santa Cruz Biotechnology; 1:500 dilution), Smad4 antibody (rabbit polyclonal IgG; Santa Cruz Biotechnology; 1:500 dilution), phospho-Smad2 antibody (rabbit polyclonal IgG; Campro Scientific, 1:1000 dilution) and glyceraldehyde-3 phosphate dehydrogenase (GAPDH) antibody (mouse-antirabbit, monoclonal IgG; Research Diagnostic inc, 1:5000), respectively. Neonatal rat cardiac fibroblasts were grown to confluence in 6-well plates. AcSDKP (0.01 to 100 nmol/l) was added 15 minutes prior to addition of TGF β 1 (5 ng/ml). Twentyfour hours later, whole cell protein was isolated by adding radioimmunoprecipitation (RIPA) buffer (constituted according to the standard protocol (Santa Cruz Biotechnology). Samples were stored at -80° C until assayed. Bands on Western blot were visualized by enhanced chemiluminescence (ECL, Amersham).

Immunocytochemical Localization of Smad Proteins

Immunocytochemistry was performed on the formalin fixed (3.7%) cells. After washing with phosphate buffer saline (PBS), ice cold methanol (100%) was added for 10 minutes, washed three times with PBS and blocked with 5% horse serum for 60 minutes. Smad2 or Smad7 antibody (1:20-1:50 in 0.1% BSA in PBS) were applied and incubated overnight at 4° C. Cells were washed with PBS, blocked with 0.1% BSA for 15 minutes, horseradish peroxidase (HRP) conjugated anti-goat polyclonal antibodies were applied for 4 hours at room temperature followed by HRP-labelled streptavidin for 60 minutes. After washing 3 times 5 minutes in PBS, DAB reagent was applied to visualize the reaction.

Statistical Analysis

All the data are expressed as mean $\pm$ SEM. One-way analysis of variance (ANOVA) followed by Student-Newman-Keuls method was used for comparing the differences among multiple groups (Instat). Significant differences among the group was defined by the probability of ≤ 0.05 .

Results

Effects of AcSDKP on serum and TGF β 1-stimulated proliferation of neonatal rat cardiac fibroblasts

Cardiac fibroblasts (plating density 120 cells/mm²) were grown until 70-80% confluent and were synchronized in medium containing 0.1% FBS, then stimulated to grow synchronously by adding either 10% FBS or 1 ng/ml TGF β 1 to the culture medium. After 24 hours of treatment of cells with and without AcSDKP, MTS reagent was added directly to the culture wells as described by the manufacturer, incubated for 4 hours and the absorbance recorded at 490 nm. Background absorbance was subtracted from the value obtained.

Addition of 10% FBS increased absorbance from 0.389 ± 0.008 to 0.445 ± 0.009 (Fig. 1a) whereas TGF β 1 increased the absorbance from 0.333 ± 0.012 (control) to 0.393 ± 0.009 (Fig. 1b). In serum-stimulated cells, co-treatment with AcSDKP (0.01 and 1 nmol/l) significantly reduced the absorbance to 0.379 ± 0.009 and 0.371 ± 0.005 ($p < 0.001$; $n = 8$) respectively (Fig. 1a). In the TGF β 1 stimulated cells, addition of 1 nmol/l concentration of AcSDKP decreased the absorbance to 0.358 ± 0.012 while AcSDKP at 0.01 nmol/l concentration did not show any effect (Fig. 1b).

To determine whether the absorbance correlates with the numbers of cells in culture, various numbers of cardiac fibroblasts were plated in 96-well plate in the medium supplemented with 10% FBS. After an hour of equilibration, MTS reagent was added directly in to the culture wells. Four hours later, absorbance was recorded at 490 nm using ELISA plate reader. The correlation coefficient was 0.96, indicating that there was a linear relation between cell number and absorbance. Background absorbance shown at 0 cells/well was subtracted from these data (Fig 1c.).

Figure 1. A. Effects of AcSDKP on growth of cultured primary neonatal rat cardiac fibroblasts stimulated by 10% FBS as shown by CellTiter 96® Aqueous One Solution Cell Proliferation Assay. Absorbance is reduced at 490 nm in the cells treated with AcSDKP 0.01 and 1 nmol/l compared to untreated cells ($M \pm SEM$; $n = 8$).

Figure 1

Figure 2

Figure 1. B. Effects of AcSDKP on the growth of cultured neonatal rat cardiac fibroblasts stimulated by TGFβ1 (1 ng/ml) and co-treated with AcSDKP (0, 0.01 and 1 nmol/l) for 24 hours. MTS reagent was added and the plate incubated for 4 hours. Absorbance was reduced at 490nm in the cells treated with AcSDKP (1 nmol/l) compared to the untreated cells ($M \pm SEM$; $n = 8$). **C.** Effects of cell numbers on absorbance at 490 nm measured using the CellTitre 96® Aqueous One Solution Assay. Various numbers of cardiac fibroblasts were plated in 96-

well plate, allowed to equilibrate for 1 hour and then the MTS reagent was added. Absorbance was recorded at 490 nm using ELISA plate reader 4 hours later. Each point represents the mean of 3 replicates.

Figure 2. Effects of AcSDKP on cell cycle status of cultured neonatal rat cardiac fibroblasts as shown by fluorescence activated cell sorter analysis. Cells stimulated with 10% FBS and treated with AcSDKP (0, 0.01 and 1 nmol/l). Flow cytometry was performed 24 hours later. AcSDKP (0.01 and 1 nmol/l) reduced G1 to S phase transition of these cells compared to the untreated cells ($M \pm SEM$; $n = 5$).

Effect of AcSDKP on transition from G1 to S phase by neonatal rat cardiac fibroblasts

To display the fluorescence signal only from living cells, scatter-gated fluorescence analysis was performed. Neonatal rat cardiac fibroblasts, either treated or untreated with AcSDKP, were detached from culture dishes by trypsin, washed with PBS and incubated with propidium iodide [50 ug/ml in 0.1% (w/v) sodium citrate/ 0.1% (v/v) triton X-100] for 15 minutes at room temperature. Flow cytometry was carried out to determine cell cycle status. In the control group $85.3 \pm 0.59\%$ of the cells were at G0/G1 phase. After 24 hours of serum stimulation, the percentage of cells in G0/G1 phase decreased to $75 \pm 1.4\%$ but when co-treated with AcSDKP (0.01 and 1 nmol/L) percentage of cells in G0/G1 phase remained $82.4 \pm 0.4\%$ and $83.7 \pm 0.5\%$ ($p < 0.05$; $n = 5$) respectively (Fig.2). Lisinopril (100 nm/l) did not have any effect on the cell cycle of cardiac fibroblast (75.9 ± 1.4 vs $75.4 \pm 1.2\%$ G0/G1 cells in 10% FBS). Amount of debris present was less than 1% in each group (see fig. 2).

Effect of AcSDKP on TGFβ1-inducible Smad binding element (SBE) promoter activity

To examine the effects of AcSDKP on TGFβ1-induced Smad signaling, we used luciferase reporter assays with the pGL3ti(SBE)4 plasmid which contains a TGFβ1 responsive element SBE and luciferase reporter gene. We first transfected pGL3ti(SBE)4 (250 ng/well) together with the control reporter pRL-CMV (25 ng/well) into cardiac fibroblasts and then continued the culture with or without TGFβ1. After 24 hours of treatment with 5 ng/ml TGFβ1, luciferase activity was elevated 6.7-fold compared to the control cells ($P < 0.01$). However, co-treatment with AcSDKP (1 nmol/L) decreased the reporter activity by $55.3 \pm 9.7\%$ ($p = 0.01$) in TGFβ1 stimulated cells (Fig. 3a). This effect was not seen in cells not stimulated by TGFβ1 (data not shown). Renilla luciferase expression was not affected by agonist treatment (control: 0.39 vs TGFβ1 0.36, TGFβ1+AcSDKP 0.35; $n = 8$, $p =$ not significant) and could, therefore, be used as an internal standard. The efficiency of the transfection reaction of neonatal rat cardiac fibroblasts was 3 pg luciferase/12000 cells indicating a high degree of transfection.

AcSDKP inhibited phosphorylation of Smad2 in TGFβ1-stimulated neonatal rat cardiac fibroblasts

Western blots were done to determine the protein concentration of Smad2, phospho-Smad2, Smad4 and Smad7 derived from cardiac fibroblasts treated with or without AcSDKP. Total Smad2 and Smad4 levels were comparable in all groups. TGFβ1 increased phospho-Smad2 levels 5.6-fold

compared to untreated cells ($p<0.01$). AcSDKP did not affect total Smad2 and Smad4 proteins (data not shown) but consistently decreased phospho-Smad2 levels in a dose-dependent manner (Fig. 3b and 3c). We were not able to detect a signal for Smad7 using the currently available antibody.

Figure 3. a. Effects of AcSDKP on TGFβ1 induced Smad binding element promoter activity. Cardiac fibroblasts were transfected with pGL3ti(SBE)4 reporter construct, then treated with TGFβ1 24 hours later. AcSDKP was added 15 minutes prior to TGFβ1. Luciferase activity was measured after 24 hours. The data obtained were expressed as the ratio of pGL3ti(SBE)4 to internal control (pRL-CMV) ($M \pm SEM$; $n=8$). **b and c,** Effect of AcSDKP on phospho-Smad2. Cardiac fibroblasts were treated with TGFβ1 and AcSDKP. After 24 hours, whole cell lysate was prepared. Western blotting was carried out with a phospho-Smad2 specific antibody ($M \pm SEM$, $n=3-4$). **b,** a representative western blot showing the effect of AcSDKP on P-Smad2 levels. Lane 1: control, lane 2: TGFβ1, lane 3: TGFβ1 + lisinopril, lane 4: TGFβ1 + lisinopril + 0.01 nmol/l AcSDKP, lane 5: TGFβ1 + lisinopril + 1 nmol/l AcSDKP, lane 6: TGFβ1 + lisinopril + 100 nmol/l AcSDKP. **c,** quantification of P-smad2 as the ratio of Smad2 in densitometric units.

Effects of AcSDKP on nuclear translocation of Smad2

Cardiac fibroblast was subjected to different doses of AcSDKP and were stimulated either or not with TGFβ1 (5 ng/ml) for 24 hours. Immunostaining for Smad2 was positive only when the cells were 50-60% confluent and were stimulated with TGFβ1. Numbers of positively stained nuclei were counted per 100 cells in 20-25 random fields per slide at 400x magnification. In control cells no nuclei stained so these were not quantified. Positive nuclear staining was seen in $72 \pm 4.9\%$ in TGFβ1 stimulated cells. When co-treated with AcSDKP (1 and 100 nmol/l), the number of nuclear

staining positive cells decreased to $58 \pm 3.4 \%$ ($p=0.08$) and $56 \pm 4.04 \%$ ($p=0.05$) respectively ($n=3$) (Fig. 3d and 3e). AcSDKP at 0.01 nmol/l concentration did not show any effect.

Effects of AcSDKP on the growth of rat aortic smooth muscle cells (PAC1)

PAC1 cells were cultured and treated similar to the cardiac fibroblasts. After 24 hours of serum deprivation, cells were stimulated to grow by supplementing them with 5% FBS with or without AcSDKP. Cells were counted 24 hours later. Addition of 5% serum caused an increase in the numbers from $24,495 \pm 3329$ cells/ml (control) to $42,375 \pm 7368$ cells/ml. Addition of AcSDKP (1 nmol/l) caused an even greater increase rising to $79,240 \pm 3488$ cells/ml. AcSDKP at 0.01 nmol/l also tended to increase the cell number but the difference was not statistically significant (Fig.4).

Discussion

AcSDKP is an important substrate inactivated by angiotensin converting enzyme. It is known to inhibit the proliferation of different cell types including cardiac fibroblasts and reduce cardiac fibrosis^{16,19,20}. Considering the important role of TGF β 1 in the induction of cardiac fibrosis, we hypothesized that AcSDKP might inhibit fibrosis via modulation of the TGF β 1 signaling cascade. We demonstrated that AcSDKP indeed inhibits the activity of Smad responsive element. Further we noted that phosphorylation as well as nuclear translocation of Smad2 was inhibited by AcSDKP.

This provides an important mechanism by which AcSDKP can inhibit TGF β signaling and thereby exert broad effects on cardiovascular cells. We also showed that AcSDKP inhibits the proliferation of cardiac fibroblasts by increasing these cells in G0/G1 phase of the cell cycle, an effect that does not depend on TGF β stimulation. Taken together, this provides a novel mechanism by which AcSDKP can inhibit both cardiac fibroblasts growth and cardiac fibroblast activity explaining its effects on cardiac fibrosis^{19,20}.

Figure 3. d. Effect of AcSDKP on Nuclear Translocation of Smad2. Cardiac fibroblasts were treated with TGFβ1 and AcSDKP. After 24 hours, cells were fixed and immunostaining carried out with Smad2 antibody. ($M \pm SEM$, $n = 3-4$). Numbers of cells positive for nuclear staining were counted per 100 cells per slide. Data represents $M \pm SEM$, $n = 3$. #, $p = 0.08$ vs. TGF β1; *, $p = 0.05$ vs. TGF β1. **e.** Representative cells showing the effect of AcSDKP on nuclear translocation of Smad2. Immunostaining was carried out on 50% confluent cells. e1: control, e2: TGF β1; e3: TGF β1 + 1 nmol/l AcSDKP, e4: TGF β1 + 100 nmol/l AcSDKP.

Figure 4. Effect of AcSDKP on growth of PAC1 cells. Cells were made quiescent by serum starvation for 24 hours. They were then stimulated to grow by supplemental 5% FBS with or without addition of AcSDKP and counted 24 hours later. AcSDKP (0.01 to 1 nmol/l) increased growth of PAC1 cells beyond the effect of 5% FBS ($M \pm SEM$, $n = 5-6$).

TGFβ1 is a cytokine that evokes its biological effects by signaling through two different receptor serine/threonine kinases, TGFβ receptor type (TβR)-I and TβR-II, that form a tetrameric complex

after binding of T β R-I to T β R-II. T β R-II activates T β R-I after phosphorylation of serine residue. This in turn leads to the activation of R-Smad. Activated R-Smads heteroligomerize with the Co-Smad4, and these complexes are translocated in to the nucleus. Once in the nucleus, the activated Smad complex binds to the promoter region of the respective gene that initiates gene transcription. Smad6 and Smad7 are classified as inhibitory Smads, which prevent R-Smad activation, thereby down regulating the TGF β signaling^{25,26}. Thus, phosphorylation of Smad2 and its subsequent translocation to the nucleus is a critical step in the modulation of cell signaling by this pathway. AcSDKP inhibited SBE promoter activity which indicated interference in Smad complex mediated gene activation. This is most likely due to either inhibition of Smad protein synthesis or inhibition of its activation and subsequent nuclear translocation, but could not definitely prove that AcSDKP inhibits phosphorylation of Smad2.

The effects of TGF β 1 on proliferation are cell-type dependent. Although TGF β 1 has been reported to arrest epithelial and endothelial cells in G1 phase,²⁷⁻²⁹ its direct effects on the cell cycle of cardiac fibroblasts are still controversial. We observed induction of proliferation of cardiac fibroblasts when cells were stimulated with TGF β 1. Although AcSDKP blunted the growth-stimulatory effect of TGF β 1, this effect could not be totally explained by inhibition of Smad2 phosphorylation. As reported previously¹⁹, we noted a biphasic response of cell growth with a decrease in the anti-proliferative effects at higher doses. On the other hand, inhibition of Smad2 phosphorylation was dose-dependent showing maximum inhibition at the highest dose we used. To explain this bell-shaped curve we postulated recruitment of inhibitory SMADs at the higher dose of AcSDKP of 100 nmol/l. As compared to Smad6, Smad7 is shown to be more sensitive to TGF β 1 stimulation³⁰. We evaluated whether AcSDKP induced Smad7. However, we were unable to detect any signal for Smad7, neither by western blot nor by immunocytochemistry. This might indicate that Smad7 expression in neonatal rat cardiac fibroblast is very low, and is not robustly induced by AcSDKP.

It has already been shown that AcSDKP inhibits the activation of extracellular signal regulated kinase (ERK) in cardiac fibroblasts,¹⁹ and we observed inhibition of serum stimulated growth of cardiac fibroblasts at doses shown to inhibit ERK activation¹⁹. Undoubtly, there is cross-talk between the TGF β 1 and ERK signaling cascade. Some studies report a positive relation between TGF β 1 and the ERK signaling pathway³¹. Others debate this finding and point out that TGF β 1 stimulates the phosphorylation of ERK and activated ERK phosphorylates the linker region of

Smad2, thereby preventing its nuclear translocation²⁵. Collectively, this suggests that AcSDKP might modify different cellular mechanisms involved in cell growth and proliferation with the net effect depending upon the interaction between these altered cellular pathways. The concentrations at which AcSDKP inhibits Smad2 activation are probably of clinical relevance during treatment with ACE inhibitors. The level of AcSDKP can increase up to 20 nmol/l during ACE inhibitor therapy¹⁶ where we would expect a clear inhibition of Smad2 phosphorylation (Fig. 3b).

TGFβ1 is known to exert an opposite effect on vascular smooth muscle cells inhibiting their growth⁹. Therefore, we postulated that if AcSDKP is indeed an important inhibitor of TGFβ1 signaling, AcSDKP should have an opposite effect on the growth of smooth muscle cells. We observed significant induction of PAC1 growth when treated with AcSDKP. This is relevant since it suggests that even though AcSDKP may beneficially inhibit cardiac fibrosis, it may have less desirable growth stimulatory effects on vascular smooth muscle cells (VSMC). This would suggest that the net effect of ACE inhibition on VSMC might be influenced by a growth-promoting effect of AcSDKP. This could explain the poorly-understood finding that ACE inhibition seemed to increase neointima formation in stented arteries³². It is clear that this effect would be most prominent in situations characterized by pure smooth muscle cell proliferation such as in-stent restenosis.

Our findings might explain the fact that ACE inhibition attenuates cardiac fibrosis even when decreased formation of angiotensin II does not seem to be involved. We recently found ‘spontaneous’ cardiac fibrosis in transgenic rats with increased ACE activity. Surprisingly, in these animals the local cardiac concentration of angiotensin II was not raised despite a 40-fold increase in ACE³³. This study suggested that a substrate of ACE other than angiotensin II could be instrumental in such condition, and our findings together with previous reports^{19,20} indicates that high ACE activity might induce fibrosis *via* excessive breakdown of AcSDKP.

In conclusion, the major finding of this study is that AcSDKP inhibits the phosphorylation and subsequent nuclear translocation of Smad2. This antagonism of TGFβ signaling provides a novel pathway by which a rise in AcSDKP (as occurs during ACE inhibition) can exert long term structural effects.

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Schematic presentation of the main findings

5

Increased myocardial collagen content in transgenic rats over-expressing cardiac ACE is related to enhanced breakdown of AcSDKP and increased phosphorylation of Smad2/3

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Abstract

Background: Although increased activity of angiotensin converting enzyme (ACE) has been associated with increased cardiac collagen, no studies to date established a direct cause-effect relation between the two. **Methods and results:** We employed transgenic rats that over-express human ACE selectively in the myocardium. Two independent heterozygous transgenic rat lines were studied, one expressing 2-3 copies (L1172) and the other expressing 5-10 copies (L1173) of the ACE transgene. These rats were normotensive but developed a proportionate increase in myocardial collagen depending on the ACE gene dose (up to 2.5-fold, $p < 0.01$) but cardiac angiotensin II levels remained normal, while collagen content reversed to control levels upon ACE inhibition. To explain these changes, we investigated N-acetyl-Ser-Asp-Lys-Pro (AcSDKP), an alternative substrate that is catabolized exclusively by ACE. Increased cardiac expression of ACE was paralleled by a reciprocal decrease in cardiac AcSDKP, and a proportionate increase in phosphorylated Smad2 and Smad3, all of which normalized following both ACE inhibition and AcSDKP infusion. Furthermore, a functional link of this signalling cascade was demonstrated as AcSDKP inhibited Smad3 phosphorylation in a dose dependent manner in cultured cardiac fibroblasts and in vivo. **Conclusion:** Our findings suggest that increased cardiac ACE activity can increase cardiac collagen content by degradation of AcSDKP, an inhibitor of the phosphorylation of TGF β signalling molecules Smad2 and Smad3. This implies that anti-fibrotic effects of ACE inhibitors are mediated in part by increasing cardiac AcSDKP, with subsequent inhibition of Smad 2/3 phosphorylation.

Introduction

Induction of angiotensin converting enzyme (ACE) has been related to cardiac fibrosis since increased expression of ACE was reported in animal models accompanied by increased cardiac collagen such as pressure-loading induced hypertrophy and myocardial infarction¹⁻³. However, it is still unknown whether the increase in cardiac ACE and increased cardiac collagen deposition are functionally linked. It has been a common assumption that pro-fibrotic effects of ACE are mediated via increased production of angiotensin (ANG) II. However, recent reports have shown that ANG II is not always elevated when cardiac collagen content is increased⁴ and tissue levels of ANG II are not lowered in homozygous ACE null mutant mice⁵, shedding doubt on this assumption.

Recent observations have suggested that excess degradation of N-acetyl-Ser-Asp-Lys-Pro (AcSDKP) can mediate the pro-fibrotic effects of ACE. AcSDKP is hydrolysed specifically by the N-terminal domain of ACE⁶. It has been previously shown that AcSDKP attenuates cardiac fibroblast proliferation and collagen synthesis both in vitro and in vivo^{7,8} and we have subsequently shown in vitro that AcSDKP directly inhibits Smad2 phosphorylation⁹ which has been confirmed in other cell types¹⁰. Recent reports have suggested that phosphorylated Smad3 (P-Smad3) is more potent regulator of collagen synthesis than P-Smad2^{11,12} but a direct relation between AcSDKP and P-Smad3 has not yet been shown in vivo.

To address a causal role for ACE in cardiac collagen production, we employed a transgenic rat model that expressed the full-length human ACE cDNA selectively in cardiomyocytes, and tested whether myocardial changes were related to altered levels of ANG II or rather AcSDKP as an alternative substrate of ACE.

Methods

Transgenic rat model and treatment protocol

We have described this model previously¹³. In brief, the full-length human somatic ACE cDNA (4.02 kb) placed under the control of a 2.1 kb rat myosin light chain-2 (MLC-2) promoter yielded two independent transgenic lines, the first with 2-3 copies (L1172) and the second with 5-10 copies (L1173) of the transgene. Activity of membrane-bound ACE was determined as reported previously¹³. The ACE transgenic rats were normotensive compared to the controls.

Since, L1173 rats expressed highest ACE-activity, we treated two sub-groups (n = 6-8 each group) of male L1173 rats aged 12-15 months either with the ACE inhibitor, lisinopril (5mg/kg/day in drinking water) for 12 weeks or with AcSDKP before sacrifice. Non-transgenic Sprague Dawley (SD) rats were used as controls. AcSDKP (Bachem Bubendorf, Switzerland) was infused at a rate of 1mg/Kg/day via subcutaneously placed miniosmotic pumps (Alzet, model 2 MI 4) for 3 weeks. All experiments were approved by the local institutional animal experiment committee.

Northern blotting

Northern blotting was done as reported previously¹⁴. The ANF probe was obtained from 825-bp BamHI fragment of the ANF cDNA cloned in pGEM-4Z (kindly provided by Dr. Sigrid Hoffmann, University of Mannheim, Germany) and TGFβ₁ probe (823-bp cDNA fragment) was generated by RT-PCR.

Histology

Myocardial collagen was stained with picosirius-red, as described earlier¹⁵. LV interstitial collagen was quantified by computerized planimetry. To measure myocyte cross-sectional area, transversely cut tissue sections were stained with anti-laminin antibody (Sigma) counterstained with hematoxylin.

Measurement of ANG II levels

For the determination of cardiac ANG II levels, heart tissue was homogenized, centrifuged and the supernatant was diluted with 0.1 M of tris-acetate buffer. The solution was passed through phenylsilica cartridges and the absorbed ANG was eluted with methanol. The elute was incubated with tracer and ANG II antiserum (a kind gift from P. Admiraal, Rotterdam, The Netherlands). Radioactive ANG II was counted by a gamma counter.

AcSDKP immunosorbant assay

AcSDKP was measured by highly sensitive and specific immunoassays¹⁶ using a commercially available kit (SPI Bio and CEA, The Netherlands). AcSDKP was isolated from LV according to the manufacturer's protocol.

Cell culture study

Cardiac fibroblasts were isolated from 2-day-old neonatal Sprague Dawley rats as described previously⁹. Cells were grown to confluence in 6-well plates and were placed in low-serum medium (0.1% FBS) for 48 hours before the experiment. The cells were then treated for 60 minutes with TGFβ1 (Sigma, Zwijndrecht, the Netherlands) with or without AcSDKP (Bachem, Bubendorf, Switzerland). Captopril (10 μM) was added to the medium to prevent degradation of AcSDKP by ACE present in the serum.

TGFβ1 receptor displacement assay

To investigate whether AcSDKP competes with TGFβ1 for its receptor binding, synchronized cardiac fibroblasts were treated with ¹²⁵I-TGFβ1 in the presence and absence of AcSDKP according to a protocol published previously¹⁷.

Protein isolation and western blotting

Protein isolation and western blotting were performed as described previously⁹. Anti-P-Smad3 antibody was a kind gift from Professor E Leof and Dr M Wilkes (Mayo Clinic Cancer Research, Rochester, Minnesota, USA), anti-collagen I/III antibodies were from Abcam and anti-TGFβ1 antibody was from Promega. All other antibodies were purchased from Cell Signaling Technologies.

Statistical analyses

Data are shown as mean ± SEM. The data were analyzed by one-way analysis of variances (ANOVA) in combination with a Dunnett post-hoc analysis to correct for multiple comparisons (SPSS 10.0). P-values of ≤0.05 were considered statistically significant.

Results

Increased ACE activity in the heart

In the left ventricle, ACE activity was increased 50-fold in L1173 and 13-fold in L1172 rats compared to controls (**Figure 1**). ACE activity in kidneys and skeletal muscles was only slightly and non-significantly increased while serum ACE was not increased in transgenic rats.

Figure 1. LV ACE activity in transgenic and control rats. L1172, transgenic rats with the lower copy number of the ACE gene (2-3 copies), L1173, transgenic rats with the higher copy number of the ACE gene (5-10 copies), *, $P < 0.01$ vs. controls, #, $P < 0.01$ vs. L1172. (Adapted from Tian et al., Human Molecular Genetics, 2004, 13 (14), 1441-1450, with permission from Oxford University Press).

Lack of increase in cardiac ANG II levels in ACE transgenic rats

Cardiac ANG II levels did not differ between transgenic and non-transgenic rats (**figure 2**). Pentobarbital was administered to a sub-group of experimental animals to investigate whether increasing renin by pentobarbital would unmask a propensity of the ACE-transgenic rats to increase their cardiac ANG II levels beyond that in controls. Pentobarbital increased renin to a similar extent in both groups (SD: 10.5 ± 2.5 , hACE transgenics: 9.4 ± 1.8 ng AngI/ml/h; $p=0.76$), and increased cardiac ANG II levels, but this increase was comparable in both transgenic and wild type rats (**Figure 2A**). Since coronary vasoconstrictor response caused by intracoronary infusion of ANG I was significantly enhanced in hACE transgenic rats, this indicated that the transgene expression was functional¹³.

Decreased cardiac AcSDKP levels in ACE transgenic rats

The myocardial concentration of AcSDKP was significantly lower in both ACE-transgenic rat lines (L1173 and L1172) compared to non-transgenic littermates. The low level of AcSDKP in L1173 normalized following lisinopril treatment (**Figure 2B**).

Figure 2. Left ventricular ANG II and AcSDKP content. **A**, LV ANG II level is not increased in ACE transgenic rats (L1173). ANG II increased similarly after pentobarbital anesthesia compared to that after isoflurane in both transgenics and controls (Adapted from Tian et al., Human Molecular Genetics, 2004, 13 (14), 1441-1450, with permission from Oxford University Press). **B**, LV AcSDKP concentration decreased in a gene-dose dependant manner while lisinopril treatment normalized AcSDKP concentration. SD = Sprague Dowley, i = isoflurane, p = pentobarbital, \$, $P < 0.01$ pentobarbital vs. isoflurane, *, $P < 0.01$ vs controls, #, $P < 0.05$ vs L1172.

Increased myocardial collagen content in ACE transgenic rats

LV collagen volume fraction (CVF) was significantly increased in 12-month old ACE-transgenic rats compared to controls which was not seen at 3-month of age¹³. Furthermore, LV CVF was also increased in the high (L1173) versus the moderate (L1172) ACE over-expression line (data not shown). Western blotting of myocardial homogenate with anti-collagen I and anti-collagen III antibody confirmed the proportional increase in cardiac collagen (**Figure 3**). Replacement of AcSDKP or treatment with lisinopril significantly decreased myocardial collagen type I and III in high ACE expressing (L1173) rats.

AcSDKP decreased Smad3 phosphorylation in cardiac fibroblasts

In synchronized cardiac fibroblasts, TGF β 1 (10 ng/ml) for 60 minutes increased Smad3 phosphorylation five-fold, which was attenuated by AcSDKP (1 and 100nM) in a dose dependant manner (**Figure 4**).

Figure 3. Myocardial collagen content in ACE transgenic and control rats. **A**, collagen type I: upper panel, representative blot, lower panel, quantification of bands in densitometric units. **B**, collagen type III: upper panel, representative blot, lower panel, quantification of bands in densitometric units. Contr, SD controls, Lis, L1173 rats treated with lisinopril, AcSD, L1173 rats treated with AcSDKP.

*, $P < 0.01$ vs control, #, $P < 0.01$ vs L1172.

Increased Smad2 and Smad3 phosphorylation in ACE transgenic rats

Both P-Smad2 and P-Smad3 were up-regulated in ACE-transgenic rats in an ACE gene-dose dependent manner compared to controls. P-Smad2 and P-Smad3 levels in L1173 rats normalized following lisinopril or AcSDKP treatment. The levels of total Smad2 and Smad3 (relative to GAPDH) were unaltered among all the study groups (**Figure 5**).

Normal TGF β 1 mRNA and protein expression in ACE transgenic rats

Cardiac TGF β 1 mRNA and protein levels were unaltered in ACE-transgenic rats with higher ACE gene expression (L1173) (**Figure 6**).

AcSDKP does not compete with TGF β 1 for receptor binding

To exclude that AcSDKP inhibits Smad signaling by competing for the TGF β receptor complex, we performed a competition assay which demonstrated that addition of AcSDKP (1nM and 100nM) did not change the amount of receptor bound 125 I-TGF β 1 in cardiac fibroblasts (controls, 499 ± 55 ; AcSDKP 100 nM, 536 ± 65 ; AcSDKP 1nM, 585 ± 98 counts per minute; $n = 7$, $P = \text{NS}$).

No left ventricular hypertrophy in ACE-transgenic rats

Heart weight to body weight ratio, myocyte cross sectional area and mRNA expression of ANF in the highest cardiac ACE expressing (L1173) rats did not differ from controls as reported previously (data not shown).

Figure 4

Figure 4. Effect of AcSDKP on P-Smad3 expression in cardiac fibroblasts. Synchronized cardiac fibroblasts were stimulated for 60 minutes with TGFβ1 with and without AcSDKP. **A**, Representative blot of P-Smad3 and total Smad3. Lane 1, control, lane 2, TGFβ1, lane 3, AcSDKP (0.01 nM), lane 4, AcSDKP (1 nM), lane 5, AcSDKP (100 nM). **B**, Quantification of P-Smad3 as the ratio of total Smad3 in densitometric units. *, $P < 0.01$ vs controls, #, $P < 0.05$ vs TGFβ1 alone, n = 3.

Figure 5

Figure 5. Total and P-Smad2 and P-Smad3 levels in untreated and lisinopril treated ACE-transgenic rats compared to controls. **A**, Upper panel, representative western-blot showing the amount of total and P-Smad2, lower panel, quantification of the levels of P-Smad2 as the ratio of total Smad2 in densitometric units. **B**, Upper panel, a representative blot showing total and P-Smad3, lower panel, quantification of P-Smad3 levels as the ratio of Smad3 in densitometric units. *, $P < 0.001$ vs. controls, #, $P < 0.01$ vs. L1172, n = 4 - 7.

Discussion

Although increased activity of ACE has been shown to be associated with cardiac fibrosis, it has remained unclear whether this association is causal. To investigate this, we characterized two lines of ACE transgenic rats with either a lower or a higher copy number of the ACE transgene to assess gene-dose effects on cardiac architecture. The main finding of this study is that cardiac collagen content increased in parallel with the ACE-gene dose, which reversed upon ACE inhibition. This increase in cardiac collagen was paralleled by a proportionate decrease in cardiac AcSDKP and an increase in phosphorylated Smad2 and Smad3 while replacement of AcSDKP reversed the phenotype.

Since ANG II levels were not elevated in ACE transgenic rats, we sought an alternative mechanism to explain the increase in cardiac collagen. We show that AcSDKP is present in the myocardium and that increased cardiac ACE decreases cardiac AcSDKP. The decrease in cardiac AcSDKP was reciprocal to the degree to which ACE was increased and was inversely related to a gene-dose dependent increase in phosphorylated Smad2 and Smad3. Furthermore, infusion of AcSDKP and ACE inhibition both normalized the levels of phosphorylated Smad2/3 and the amount of collagen I and III. Taken together, we demonstrate that a primary and selective increase of cardiac ACE activity results in increased cardiac collagen deposition, which we suggest to be caused by excessive breakdown of cardiac AcSDKP. The decrease in AcSDKP could release the inhibitory effects of this peptide on phosphorylation of Smad2 and Smad3.

Relation between ACE, ANG II and collagen content

An increased level of ACE has been associated with excess production of extra-cellular matrix in the heart, which was presumed to be mediated via increased ANG II. We did not find an increase in cardiac ANG II, which underscores that ACE is not the rate-limiting step in the formation of ANG II. In agreement, Perry et al.¹⁸ have shown that cardiac ANG II levels remained unaltered despite differences in ACE genotype and ACE expression in mice. The lack of rise in ANG II cannot be explained by a lack of interaction between the over-expressed enzyme with intracoronary ANG I as we have described increased ACE-dependent conversion of intracoronary ANG I in the isolated perfused hearts of ACE transgenic rats¹³.

AcSDKP is an anti-fibrotic agent

AcSDKP is a specific substrate of ACE and its level increases substantially following ACE inhibitor therapy¹⁹. Although the functional role of exogenous AcSDKP was demonstrated in the myocardium^{7,20}, endogenous levels of this peptide in the myocardium had not yet been shown. We show here that cardiac AcSDKP levels in the myocardium of wild type rats is higher than what has been reported in plasma, suggesting that the myocardium actively generates or traps AcSDKP.

The cardiac concentration of AcSDKP inversely correlated with the level of myocardial ACE over-expression. Following ACE inhibitor therapy, the level of AcSDKP increased profoundly in ACE transgenic rats reaching the level obtained in non-transgenic controls. Previous studies have suggested an important role for AcSDKP in cardiac matrix regulation. AcSDKP was shown to reduce DNA and collagen synthesis in cardiac and renal fibroblasts^{8,21}. Moreover, AcSDKP decreased cardiac and renal fibrosis in 2-kidney, 1-clip hypertensive as well as aldosterone-salt hypertensive rats^{7,20}. We found that the levels of AcSDKP inversely correlated with cardiac collagen content in ACE-transgenic rats. Despite the fact that AcSDKP was infused only for 3 weeks, AcSDKP-treated ACE-transgenic rats had substantially lower levels of collagen I and III compared to the untreated transgenic controls. This relatively rapid decrease in collagen indicates that apart from its inhibitory role in collagen synthesis, AcSDKP may also stimulate collagenolysis possibly via independent mechanisms such as activating collagenases in a direct or indirect way.

Figure 6. TGFβ measurements in myocardial homogenate. **A**, mRNA expression of TGFβ1 in the myocardium of L1173 rats compared to wild type controls (n=7). **B**, protein levels in controls and transgenic rats (n=3 each), upper panel shows a representative blot corresponding to the bar.

Relation between TGFβ1, Smad2 and AcSDKP

TGFβ1 stimulates the deposition of collagen leading to an increased myocardial collagen content during cardiac hypertrophy and failure^{22,23}. This pro-fibrotic effect of TGFβ1 is importantly conveyed by Smad proteins, which are phosphorylated and then translocate to the nucleus to initiate gene expression.

We have recently shown that AcSDKP directly inhibits Smad2 phosphorylation in isolated cells⁹. Here, we observed that AcSDKP also inhibits TGFβ1 mediated phosphorylation of Smad3 in cardiac fibroblasts. Moreover, infusion of AcSDKP in ACE-transgenic L1173 rats normalized the levels of P-Smad2 and 3 in the myocardium. Taken together, AcSDKP appears to be an endogenous inhibitor of Smad signaling. Therefore, ACE may induce myocardial collagen production via breakdown of AcSDKP and the subsequent release of inhibition on Smad2/3 phosphorylation. Smad2 and 3 are reported to be elevated in various models of myocardial fibrosis and inhibition of Smad2/3 phosphorylation by angiotensin receptor blockers (AT1 blockers) has been shown to reduce post-infarct fibrosis^{4,24,25}. An AT1 blocker could possibly inhibit myocardial collagenesis also in ACE transgenic rats by this mechanism. In our study, we found up-regulated levels of P-Smad2/3 in ACE-transgenic rats while the mRNA expression of TGFβ1 was not elevated. It is striking that the direct effects of AcSDKP in vitro and in vivo on both Smad2 and Smad3 are highly similar, suggesting a common mode of action of AcSDKP in inhibiting Smad2 and 3 phosphorylation. In isolated cardiac fibroblasts, AcSDKP inhibited Smad3 phosphorylation within one hour. This indicates that de-novo synthesis of an inhibitory protein or inhibition of synthesis of an excitatory molecule of Smad phosphorylation is less likely. These findings suggested that AcSDKP may be a competitive inhibitor of the TGFβ receptor complex, but a ligand displacement assay did not support that idea. Therefore, the precise mechanism by which AcSDKP inhibits Smad phosphorylation remains elusive.

It has been shown that over-expression of Smad7, an inhibitory Smad protein, decreased collagen synthesis in adult cardiac fibroblasts and Smad7 expression was decreased in infarct scar²⁶. However, in neonatal cardiac fibroblasts, the levels of Smad7 was undetectable and we did not find any effect of AcSDKP on Smad7⁹. Still, as we have not measured Smad6/7 in the myocardium of

ACE-transgenic rats, the possible effects of ACE on the expression of these inhibitory proteins cannot be excluded.

Apart from ANG I and AcSDKP, ACE catalyzes the breakdown of several substrates including ANG 1-7 and bradykinin. Although bradykinin is shown to be related to cardiac fibrosis²⁷, a relationship between Smad2/3 phosphorylation and bradykinin is not yet established. Recently it was demonstrated that ANG 1-7 is localized selectively in the myocytes whereas fibroblasts and other collagen-containing cells were devoid of ANG 1-7²⁸. This suggested that the decrease in the breakdown of ANG 1-7 may have less effect on cardiac collagen production in ACE-transgenic rats. Nevertheless, we cannot rule out the effects of excess degradation of these molecules in our transgenic model.

In summary, this study shows that AcSDKP is present in the myocardium in biologically relevant concentrations. Increased cardiac ACE decreased cardiac AcSDKP in vivo in a gene-dose dependent manner, which was paralleled by increased phosphorylated Smad2 and Smad3 as well as increased cardiac collagen content suggesting that increased cardiac ACE degrades AcSDKP in sufficient amounts to release the inhibition of Smad2/3 phosphorylation and induce an excess deposition of collagen. ACE-inhibitors may interfere with this process by preventing the degradation of AcSDKP. This indicates that the maintenance of adequate AcSDKP concentrations in the heart may be one of the major factors mediating the beneficial structural effects of ACE inhibition.

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Schematic presentation of the main findings

6

Cardiomyocyte-specific deletion of the type I TGF β receptor prevents angiotensin II induced P38 activation, cardiac hypertrophy and dysfunction

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Abstract

Background: TGF β signalling in the heart has often been suggested to be important in the development of myocardial hypertrophy, fibrosis and dysfunction. TGF β and its receptors are found on both cardiac myocytes and fibroblasts, and TGF β signalling includes not only activation of Smad but also P38 mitogen activated protein kinase (MAPK). However, role of TGF β receptors (TGF β R) on myocytes in the development of cardiac hypertrophy is unclear. Furthermore, which signalling cascade is predominantly related to myocytic-TGF β receptor function *in vivo* is also not established. We addressed the development of cardiac hypertrophy in a model by using cre-loxP system to inactivate the TGF β R type I (TGF β RI) specifically in the cardiomyocytes of adult mice.

Methods and results: Mice expressing tamoxifen sensitive *cre* under the control of the α -MHC promotor were crossed with homozygous TGF β RI floxed (TGF β RI^{fl/fl}) mice to generate TGF β RI^{fl/cre} mice. To induce *cre*-recombination, tamoxifen was injected 3 weeks before operation to create myocyte-specific TGF β RI^{-/-} mice (TGF β RI^{-/-}). We infused AngII (0.5mg/kg/day) for 4-weeks in TGF β RI^{+/+} and TGF β RI^{-/-} mice. Age, sex and genotype-matched saline treated mice were used as controls. In TGF β RI^{+/+} mice, AngII induced myocardial hypertrophy and impaired ventricular ejection fraction, fractional shortening, systolic and diastolic dP/dT while these effects of Ang II were absent in TGF β RI^{-/-} mice. Western blotting revealed that Ang II induced a robust increase in phosphorylated-P38 in mTGF β RI^{+/+} mice but not in TGF β RI^{-/-} mice. However, Ang II induced Smad2 phosphorylation in both mTGF β RI^{+/+} and TGF β RI^{-/-} mice. *Conclusion:* These results show that signalling through TGF β RI in cardiomyocytes is a crucial pathway for Ang II induced cardiac hypertrophy and dysfunction. This effect of the cardiomyocytic TGF β RI seems to be mediated by p38 MAPK, rather than by Smad signalling.

Introduction

While increased cardiac loading by hypertension induces left ventricular hypertrophy, the simultaneous activation of various growth factors initiate cellular changes that progressively deteriorate cardiac function ultimately culminating to overt heart failure¹. Angiotensin II is known to increase blood pressure, induce cellular hypertrophy and further directly recruits molecules like TGF β 1². Ang II-mediated increase in TGF β have been shown to correlate with cardiac hypertrophy³, fibrosis^{4,5}, and recapitulation of fetal isoforms of cardiac myofibrillar proteins⁶. These multiple lines of evidence have suggested that cardiac recruitment of TGF β and its mediators may be pivotal in the development of cardiac hypertrophy and its adverse sequels. However, definitive evidence to conclude whether TGF β is indeed crucial for the development of cardiac hypertrophy and dysfunction and, which signaling cascade related to TGF β is involved in this process is still lacking.

A TGF β 1 neutralizing antibody has been shown to prevent cardiac fibrosis and diastolic dysfunction in mice after aortic banding but in this study no effect was seen on cardiac hypertrophy⁷. In TGF β 1 deficient mice, Ang II infusion did not induce cardiac hypertrophy but no effect was noted on cardiac collagen content⁸. However, in this somatic gene inactivated model, the widespread loss of a pleiotropic agent, TGF β , may have had an alterations in multi-system functions that could influence the outcome of the study. This has been exemplified by the fact that these mice die in early post-natal life due to widespread autoimmune-inflammation⁹, so that these TGF β 1 null mice needed to be bred in an immunocompromised *rag* null background to keep them alive till adulthood.

TGF β 1 and its receptors are found in various cells in the myocardium including cardiac myocytes, fibroblasts and other cardiac cell types¹⁰. It has been shown that over-expression of TGF β activated kinase1 (TAK1) and subsequent increase in P38 activity in cardiac myocytes induced hypertrophy and heart failure¹. However, whether disruption of TGF β -P38 axis is sufficient to prevent cardiac hypertrophy and dysfunction is still unknown.

We hypothesized that activation of TGF β signaling specifically in cardiac myocytes is necessary to promote Ang II induced cardiac hypertrophy and failure. Therefore, we generated mice where we

could inactivate the TGF β type I receptor (TGF β RI) specifically in cardiomyocytes. We crossed MerCreMer transgenic mice expressing tamoxifen-inducible *cre* fusion protein under the control of an α -myosin heavy chain (MHC) promoter¹¹ with mice having TGF β RI targeted with Lox-P sites¹² that enabled inactivation of TGF β RI gene in cardiomyocytes in a time controlled manner.

Methods

Generation of double transgenic mice: TGF β RI^{*fl/fl*} mice (C57Bl/6 background) generated by flanking exon 3 of TGF β RI with lox-P site¹² were crossed with mice (C57Bl/6 FVB background) containing *cre*-recombinase under the control of α -MHC promoter (MerCreMer^{*cre/wt*})¹¹ to generate heterozygous double transgenic mice that contained TGF β RI^{*fl/wtcre*} genes. These mice were then back-crossed with TGF β RI^{*fl/fl*} mice resulting in a colony with TGF β RI^{*fl/flcre*} and TGF β RI^{*fl/wcre*} in a mixed background of C57Bl/6 FVB, and C57BL/6. Mice were handled in accordance with standard use protocols and animal welfare regulations approved by the Institutional Animal Care and Use Committee, Maastricht University.

DNA isolation and genotyping

DNA was isolated from the mouse tail using genomic DNA purification kit (Promega) according to the manufacturer's instruction. We used PCR to assess the genotype of T β RI flox mice, using the 3 primers, 5'-ATG AGT TAT TAG AAG TTG TTT, 3'-ACC CTC TCA CTC TTC CTG AGT, and 3'-GGA ACT GGG AAA GGA GAT AAC as previously described¹². For the detection of the MerCreMer transgene, we used primers 5'-CCT GGA AAA TGC TTC TGT CCG and 5'-CAG GGT GTT ATA AGC AAT CCC, which amplifies a 400-bp fragment from *cre*.

Cre recombination protocol

To induce α -MHC-coupled *cre* recombinase in cardiomyocytes, adult TGF β RI^{*fl/flcre*} and TGF β RI^{*fl/wtcre*} double transgenic mice were treated with tamoxifen (Sigma) at a dose of 20 mg/kg per day for 7 days by subcutaneous insertion of mini-osmotic pumps (ALZET, model 2001). A group of wild type mice was treated with tamoxifen to check whether tamoxifen itself had any effects on cardiac morphology and function. Tamoxifen was dissolved in 10% ethanol and 90% polyethyleneglycol-400 followed by a brief sonification. Mice were allowed to recover for 2-week prior to treatment with Ang II or vehicle.

Subcutaneous implantation of mini-osmotic pumps and Ang II infusion

Mice of either sex weighing 24-32 g were anesthetized with 2.5% isoflurane. Under sterile conditions, a midscapular incision was made, a pocket was created in the subcutaneous tissue by a blunt dissection and a mini-osmotic pump (ALZET model 2004; ALZACorp., Palo Alto, California, USA) filled with saline or Ang II (0.5mg/kg/day) was inserted. The contents of the mini-osmotic pump were delivered into the local subcutaneous space at a rate of 0.25 μ l/hour for 4 weeks. In each group, 7 to 9 mice were recruited for experiments and at least 5 mice from each group completed the experiments. All the dropouts were due to death from anaesthesia except 3 animals in which LV catheterisation did not succeed.

Echocardiography

Transthoracic echocardiography was performed preoperatively and after 4-week of angII infusion in wild type, *TGF β RI* $-/-$ and *TGF β RI* $^{-/+}$ mice under 2.5% isoflurane anaesthesia. Standard views were obtained in 2D-echocardiography, end-diastolic and end-systolic internal diameters were measured and ejection fraction and fractional shortening were calculated.

Hemodynamic measurements

Mice were anaesthetized with intraperitoneal injection of urethan. A Millar (1.4F) catheter (Millar Instruments Inc., Houston, Texas, USA) was placed in the right common carotid artery and advanced into the left ventricle for the measurement of left intraventricular pressure. Body temperature was maintained at 37°C using a thermally controlled surgical table and monitored with a rectal probe. The mice were then allowed to stabilize for 30 minutes prior to hemodynamic measurements.

Tissue procurement and myocardial morphometry

Following hemodynamic measurements, hearts were rapidly excised, washed in 0.9 % sodium chloride solution, atria were removed and the ventricles were cut into pieces. For RNA and protein isolation, samples were snap frozen in liquid nitrogen and stored in -80 °C. For histological analysis, left ventricles were fixed in paraformaldehyde (1%) and embedded in paraffin. For the visualization of total collagen, picrosirius staining was performed as described previously¹³. P38 was localized by immunostaining using anti-P38 antibody according to the manufacturer's instruction (Cell Signaling Technology, Leusden, the Netherlands).

Protein isolation and western blotting

Frozen ventricles were crushed and homogenized in radioimmunoassay buffer according to the standard protocol (SantaCruz Biotechnology, Leiden, the Netherlands). Western blotting was performed using specific antibodies against T β RI (1: 1000), total and phospho (P)-Smad2, total and P-P38 (1:1000, Cell Signaling Technology, Leusden, the Netherlands), P-Smad3 (1: 5000, a kind gift from Professor E. Leof and Dr M. Wilkes, Mayo Clinic Cancer Research, Rochester, Minnesota, USA), collagen I (1:3000) and III (1:500) antibodies (Abcam, Leusden, the Netherlands).

Statistical analyses

Data are shown as mean $\pm$ SEM. Unpaired t-test was performed to compare the difference between the means of Ang II and vehicle treated animals from the same genetic strain. P-values of ≤ 0.05 were considered statistically significant.

Results

Generation of cardiomyocyte-specific conditional TGF β RI^{-/-} mice

Following tamoxifen treatment, we measured the levels of mature TGF β RI in the myocardium to assess the effects of *cre*-mediated deletion of *TGF β RI* gene. We found a 58 ± 10 % reduction in the T β RI signal in myocardial homogenates from *TGF β RI*^{-/-} mice compared to the wild type controls (Figure 1). In contrast, we found only a 3% reduction of *TGF β RI* in *TGF β RI*^{*wt/cre*} mice.

To investigate whether tamoxifen injection affected myocardial structure and function, a subgroup of wild type (n = 3) and TGF β RI^{*wt/cre*} mice (n = 5) were treated with tamoxifen for 1 week. Echocardiography, hemodynamics and gravimetric analysis showed no difference in LV size and functions between tamoxifen treated and untreated mice of both genotypes (data not shown).

Preserved ventricular dimensions and functions in TGF β RI^{-/-} mice

Ang II significantly decreased left ventricular ejection fraction and fractional shortening in wild type and TGF β RI^{-/+} mice (data only shown for wild type mice) while these effects were absent in TGF β RI^{-/-} mice compared to their vehicle treated controls. Similarly, left ventricular diameters did not differ between the Ang II treated and saline treated TGF β RI^{-/-} mice (Table 1). Ang II infusion did not have any effect on the rate of ventricular contraction (+ dP/dt) and relaxation (-dp/dt) in

TGF β RI^{-/-} mice in contrast to the significant deterioration of cardiac function in TGF β RI^{+/+} littermates (Table 2).

Lack of Ang II-induced myocardial hypertrophy in TGF β RI^{-/-} mice

Ang II significantly increased interventricular septal thickness (Table 1), heart weight and ventricular weight compared to their age and sex-matched saline-treated controls in TGF β RI^{+/+} mice, while no such effects were observed in TGF β RI^{-/-} mice (Figure 2).

Figure 1. Effects of tamoxifen on cre-recombination in TGF β RI^{fl/crc} TGF β RI^{fl/wtcre} mice compared to wild type controls. A, a representative immunoblot for TGF β RI. B, quantification of TGF β RI bands normalized to GAPDH. * $P < 0.05$ vs TGF β RI^{+/+}.

Figure 2. Measurements of LV weight following Ang II treatment. The ratio of LV weight and to tibia length in TGF β RI^{-/-} and wild type mice. There was no significant difference between Ang II and vehicle-treated TGF β RI^{-/-} mice. * $P < 0.05$ vs. TGF β RI^{+/+} placebo.

LV collagen content not decreased in TGF β RI^{-/-} mice

We stained ventricular sections of Ang II treated and control animals by picrosirius staining to visualize total collagen. We noted a mild increase in perivascular as well as interstitial fibrosis in the left ventricles of the Ang II treated animals (not shown). To quantify and specify the type of collagen, we performed western blot to detect type I and III collagen in the myocardial homogenate. In wild type mice, Ang II significantly increased both collagen type I and type III compared to the controls. Similarly, in TGF β RI^{-/-} mice, both types of collagens were increased at a discernible levels in response to Ang II but the difference with placebo treated controls were not statistically significant (figure 3 A and B).

Figure 3. Measurements of left ventricular collagen content in TGF β RI^{+/+} and TGF β RI^{-/-} mice.

A, quantification of LV collagen I, upper panel shows a representative blot of collagen I. **B**, levels of LV collagen III, upper panel shows a representative blot of collagen III. GAPDH blots demonstrate equal protein loading. No significant difference is noted on collagen content between Ang II and vehicle treated TGF β RI^{-/-} mice, $n = 4-5$ each group, * $P < 0.05$ vs. wild type controls.

Blunted P38 activation in TGF β RI^{-/-} mice

Since P38 has been shown as an effector for TGF β mediated cardiac hypertrophy¹, we tested the phosphorylation status of P38 in the myocardial homogenates. Phosphorylated-P38 was increased 20-fold after Ang II infusion in TGF β RI^{+/+} mice compared to the placebo-treated controls. In contrast, Ang II failed to increase Phosphorylated -P38 in TGF β RI^{-/-} mice (Figure 4).

Table 1. Comparison of left ventricular echocardiographic parameters in Ang II and saline treated TGF β RI^{+/+} and TGF β RI^{-/-} mice.

	Ejection fraction	Fractional shortening	LVIDd	LVIDs	IVSd
TGFR ^{+/+} placebo	63.2 $\pm$ 2.0	31.8 $\pm$ 1.0	0.33 $\pm$ 0.02	0.20 $\pm$ 0.01	0.125 $\pm$ 0.01
TGFR ^{+/+} Ang II	44.7 $\pm$ 2.2*	19.1 $\pm$ 0.9*	0.32 $\pm$ 0.01	0.26 $\pm$ 0.01*	0.148 $\pm$ 0.01*
TGFR ^{-/-} placebo	64.5 $\pm$ 4.1	32.2 $\pm$ 1.7	0.34 $\pm$ 0.04	0.23 $\pm$ 0.01	0.136 $\pm$ 0.02
TGFR ^{-/-} Ang II	65.4 $\pm$ 3.3	35.8 $\pm$ 2.2	0.35 $\pm$ 0.01	0.22 $\pm$ 0.01	0.132 $\pm$ 0.01

LVP, left ventricular pressure, LVIDd, left ventricular internal diameter in diastole, LVIDs, left ventricular internal diameter in systole, IVSd, interventricular septal thickness in diastole *, P < 0.05 vs. TGF β RI^{+/+} placebo

Table 2. Comparison of hemodynamic parameters after 4-week infusion of Ang II or normal saline in TGF β RI^{+/+} and TGF β RI^{-/-} mice

	LVP _{sys} (mmHg)	LVP _{dias} (mmHg)	DP/dT _{max} (mmHg/sec)	DP/dT _{min} (mmHg/sec)
TGFR ^{+/+} placebo	88.8 $\pm$ 5.5	1.6 $\pm$ 1.6	8697 $\pm$ 518	5980 $\pm$ 434
TGFR ^{+/+} Ang II	71.4 $\pm$ 5.1	9.4 $\pm$ 6.5	4644 $\pm$ 481*	3536 $\pm$ 409*
TGFR ^{-/-} placebo	76.3 $\pm$ 7.1	1.9 $\pm$ 0.9	9847 $\pm$ 1487	5505 $\pm$ 417
TGFR ^{-/-} Ang II	68.6 $\pm$ 5.4	-1.8 $\pm$ 1.6	7144 $\pm$ 718	5661 $\pm$ 247

LVP, left ventricular pressure, *, P < 0.05 vs. TGF β RI^{+/+} placebo

P-Smad2 not decreased in TGF β RI^{-/-} mice

P-Smad2 levels were increased in Ang II-treated TGF β RI^{+/+} mice compared to the saline treated controls. In TGF β RI^{-/-} mice, Smad2 phosphorylation was increased but the difference with placebo-treated controls were not statistically significant (**Figure 5**).

P38 localized in cardiomyocytes

To identify the main site of P38 and Smad2 action, we performed immunohistochemistry on the tissue sections with anti-P38 antibody and anti-Smad2 antibody. We observed a positive staining for P38 predominantly in the cardiomyocytes while Smad2 was localized in both myocytes and non-myocytes (**Figure 6**).

Figure 6

Figure 4. Measurements of LV P-P38 presented as the ratio of P38 in wild type and TGFR^{-/-} mice treated with and without Ang II. Upper panel shows a representative blots. * $P < 0.05$ vs. TGFR^{+/+} placebo, $n = 4-5$ each group.

Figure 5. Levels of P-Smad2 in Ang II and vehicle-treated mice. The data are presented as the ratio of phospho vs. total Smad2. Upper panel shows representative blots. * $P < 0.05$ vs. TGFR^{+/+} placebo, $n = 4-5$ each group.

Figure 6. Immunohistochemical localization of P38 and Smad2 in myocardial section of wild type mice. A, localization of P38 predominantly in myocytes. B, Smad2 localized in both myocytes and nonmyocytes.

Discussion

The major finding of this study is that the myocytic TGF β RI is necessary for the Ang II-induced hypertrophic response and progression to cardiac dysfunction. We used an inducible cre/loxP system to temporally inactivate the TGF β RI selectively in cardiomyocytes of adult mice to circumvent the complications caused by an early and generalized loss of TGF β signalling that results from a somatic gene inactivation strategy. Both echocardiographic and hemodynamic measurements demonstrated that Ang II infusion at a dose of 0.5 mg/Kg/day induced left ventricular hypertrophy and cardiac dysfunction in wild type mice while cardiomyocytic inactivation of the TGF β RI prevented left ventricular hypertrophy and preserved left ventricular function. However, Ang II increased total myocardial collagen in both genotypes. Molecular analyses revealed that Ang II induced phosphorylated-P38 in TGF β RI^{+/+}, but that this response was absent in TGF β RI^{-/-} mice. These data suggest that the myocytic TGF β RI is necessary to mediate the adverse sequels of Ang II and high blood pressure and that rather than the well known Smad signalling system, the myocytic Ang II- TGF β RI axis employs P38 MAPK as an essential hypertrophic mediator.

Analysis of TGF β function in vitro and in vivo

TGF β 1 has been thought to be a candidate mediator for the cardiac remodeling induced by Ang II since Ang II is shown to increase expression of TGF β protein not only in cultured cardiac myocytes and fibroblasts, but also in vivo^{3-6,13,14}. A number of studies have shown that mRNA levels of TGF β 1 markedly increased in the myocardium upon pressure loading or following pharmacological manipulations and that these mRNA changes correlated to hypertrophy and fibrosis^{13,15}.

Kuwahara and colleagues reported that aortic banding in rats induced myocardial hypertrophy, collagen deposition and TGF β 1 mRNA expression, and that treatment with a TGF β 1 neutralizing antibody decreased collagen content but failed to improve ventricular size⁷. In an another study, loss of TGF β 1 prevented Ang II induced myocardial hypertrophy but did not influence cardiac fibrosis⁸. Since conventional TGF β 1 knock out mice die before weaning due to wide-spread autoimmune like inflammation, Schultz et al. used TGF β 1 knock out mice bred in immunocompromised rag null background⁸. Although somatic loss of TGF β yielded a clear effect, these results can importantly be confounded in two ways. Firstly, since TGF β 1 signaling is essential

in many cell types, the somatic loss of TGF β signaling can affect multiple systems. For instance the loss of TGF β 1 may negatively affect angiogenesis¹², which could readily affect the outcome of this study. Secondly, at least a part of the effects of Ang II in the hearts are attributed to its pro-inflammatory actions that results into infiltration of inflammatory cells and associated cytokines in the myocardium¹⁶. The use of immunocompromised animals precludes this part of Ang II-induced changes in cardiac morphology and functions, limiting the utility of this model. Furthermore, study by Schultz et al. has not analysed which of the signalling cascades related to TGF β 1 was involved in Ang-mediated cardiac hypertrophy.

Role of the cardiomyocytic TGF β type I receptor

It has previously been shown that an activating mutation of TGF β RI coupled to α -MHC promoter disrupts cardiac morphogenesis, with invariable death before embryonic day- 12¹⁷. Conversely, deletion of exon 3 of the TGF β RI also leads to embryonic lethality¹² obviating the possibility to study the adult animals. To circumvent these problems, we generated a mouse model where the TGF β RI can be disrupted in a temporal and tissue specific manner. To enable inactivation of TGF β RI gene in cardiomyocytes in a time controlled manner, we employed MerCreMer transgenic mice expressing tamoxifen-inducible *cre* fusion protein under the control of an α -MHC promoter¹¹ which has been extensively characterized and shown to (in)activate targeted gene specifically in cardiomyocytes and not in other cell types and tissues like brain, kidney, lung, liver, and skeletal muscle¹¹. It has been shown that essentially no *cre*-mediated recombination occurred in the embryonic, neonatal, or adult heart in the absence of inducing agent but >80% recombination occurred after tamoxifen injections. Expression of the MerCreMer fusion protein within the adult heart did not affect cardiac performance, cellular architecture, or expression of hypertrophic marker genes, demonstrating that the transgene-encoded protein is relatively innocuous¹¹.

TGF β RI^{*ffcre*} mice were born without clear abnormalities and appeared healthy into adulthood indicating that the floxed TGF β RI allele functioned normally in the presence of *cre*. The levels of mature TGF β RI in cardiac tissue of tamoxifen untreated TGF β RI^{*ffcre*} mice were not different from the tissue of wild type mice (data not shown) indicating that the tamoxifen inducible α -MHC promoter was not spontaneously activated. Echocardiography demonstrated that neither cardiac morphology nor cardiac function was altered by the presence of the MerCreMer transgene that goes in agreement with the earlier report¹¹.

It is known that the TGF β RI is located on many different cell types, including cardiomyocytes and nonmyocytes in the heart. The current data indicate that the cardiomyocytic TGF β RI are essentially involved in the development of LV hypertrophy and ultimate failure. Interestingly, the loss of the cardiomyocytic TGF β RI not only affected myocardial hypertrophy and systolic function, but also reduced collagen I and III to some extent indicating an important role for cardiomyocytic TGF β RI signalling in the extracellular matrix regulation.

TGF β type I receptor signalling

The signals from TGF β 's are relayed by cell surface transmembrane receptors, TGF β RI and TGF β RII. Ligand binding induces TGF β RII receptor to associate with TGF β RI leading to a unidirectional phosphorylation of TGF β RI thereby activating its kinase domain^{18,19}. Activated TGF β RI phosphorylate multiple intracellular targets like Smads and MAPKs, which translocate to the nucleus to initiate transcription of specific genes^{20,21}. In the myocardium, activation of Smad2 and Smad3 has mainly been implicated in extracellular matrix turn over. In a rat model of myocardial infarction, increased synthesis and phosphorylation of Smad2/3 and Smad4 was seen in scar tissue and its blockade decreased myocardial collagen content^{22,23}. Similarly, increased Smad2 synthesis was associated with myocardial fibrosis in cardiomyopathic hamsters²⁴. Strong association between Smad and collagen synthesis has been reported in many other in vitro and in vivo studies as well²⁵⁻²⁸. Smad3 knock out in hepatic stellate cells resulted in marked reduction in CCL4 induced fibrosis whereas no changes were noted in α -smooth muscle actin levels²⁶. In the current study, Ang II-induced Smad2 phosphorylation and collagen deposition to a variable degree in TGF β RI^{+/+} and TGF β RI^{-/-} mice. Immunohistochemistry showed that Smad2 was localized in both myocytes and nonmyocytes. Therefore, relatively lesser degree of P-Smad2 in Ang II-treated TGF β RI^{-/-} mice may be due to a loss of Smad2 phosphorylation in myocytes. This indicates that activation of Smad2 regulates matrix related genes in both myocytes and non-myocytes.

Besides the activation of Smad proteins, TGF β 1 can activate other signalling molecules like P38 through the activation of upstream kinase activator, TAK1. Recently, Yu and associates reported that the TGF β RI is essential for TGF β 1 mediated activation of P38, which was independent of Smad phosphorylation²¹. They demonstrated that TGF β dependent activation of P38 and Smad2 mediate two distinct functions, since P-P38 was required for TGF β mediated apoptosis and epithelial to mesenchymal transition while Smad phosphorylation was necessary for growth arrest.

A large body of evidence has linked P38 activity to myocyte hypertrophy and heart failure. In cultured neonatal myocytes, over-expression of MKK3/MKK6, upstream kinases activating P38, induced hypertrophy and ANF expression²⁹ and P-P38 mediated endothelin induced myocyte hypertrophy³⁰.

Zhang and colleagues demonstrated that TAK1 transgenic mice developed myocardial hypertrophy and cardiomyopathy that was attributed to the activation of P38 in the hearts¹. Similarly, activation of P38 was implicated as the primary mechanism of myocardial hypertrophy due to aortic banding¹. In PDK3 deficient mice, level of P-P38 was attenuated, which was accompanied by thin walled small hearts indicating that P-P38 is necessary for myocyte growth³¹. In line with these observations, we found that Ang II induced cardiac hypertrophy and dysfunction was accompanied by a robust increase in P-P38 in wild type mice. In contrast, Ang II completely failed to induce phosphorylation of P38 and LVH in TGF β RI^{-/-} mice. Immunohistochemistry revealed that P38 was localized predominantly in the cardiomyocytes. Taken together, this strongly suggests that P38 MAPK is the main down stream effector of TGF β RI signaling in cardiomyocytes. One may argue that besides TGF β , P38 can be activated by other systems like a direct stimulation of G-proteins by the AT1 receptor. However, the complete lack of activation of P38 in Ang II treated TGF β RI^{-/-} mice indicates that intact TGF β RI signalling is essential for Ang II-induced P38 activation. This implies that blockade of TGF β RI may beneficially interfere with the hypertrophic response of the cardiac myocyte, and preserve cardiac function.

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Schematic presentation of the main findings

7

Stretch-induced connective tissue growth factor expression in cardiac fibroblasts is mediated by EGF-receptor-coupled β 1 integrin

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Abstract

Background: Excess collagen accumulation marks adverse left ventricular (LV) hypertrophy. Connective tissue growth factor (CTGF) is an important mediator of collagen accumulation. Neurohormones such as angiotensin II can increase CTGF expression. However, the role of mechanical stretch on CTGF regulation is unclear. We studied whether mechanical stretch increases CTGF levels in cardiac fibroblasts, and whether inhibition of CTGF can attenuate collagen synthesis in pressure-overloaded hearts. *Methods and results:* Equi-biaxial stretching of cardiac fibroblasts significantly increased CTGF levels in a time dependent manner. An anti- $\beta 1$ integrin antibody abolished this increase in CTGF. Treatment with the selective inhibitor of phosphorylation of the EGF receptor - tyrphostin AG1478, also abolished the CTGF increase following stretch and diminished basal CTGF levels. However, stimulation of the EGF receptor with its ligand EGF did not alter CTGF levels neither under basal conditions nor after stretch. In homozygous hypertensive m(Ren2)27 transgenic rats, cardiac collagen content was increased in parallel with increased cardiac CTGF levels. Chronic treatment for 4 weeks of these Ren2 rats with the inhibitor of phosphorylation of the EGF receptor, tyrphostin A46, decreased LV end diastolic pressure and LV collagen volume fraction and decreased CTGF levels. *Conclusion:* Our study shows that stretching cardiac fibroblasts increases CTGF. This effect of mechanical stretch is mediated by a $\beta 1$ integrin dependent activation of the EGF receptor. This suggests that inhibition of the $\beta 1$ integrin - EGF receptor cascade may prevent increased CTGF production, which may prevent cardiac collagen accumulation and LV dysfunction in chronically overloaded hearts.

Introduction

Matrix accumulation, collagen deposition and stiffening hallmark the adverse remodeling in chronically overloaded myocardium¹. The underlying mechanisms are not yet fully elucidated, but neurohormones and growth factors such as angiotensin II (ang II), endothelin and TGF β are associated with enhanced synthesis of excess collagen and other matrix proteins and have been implicated in high blood pressure and heart failure²⁻⁴. In addition, accumulating evidence suggests that collagen is also produced in direct response to mechanical loading itself as a means of withstanding increased mechanical force. For example, it has been shown that fibroblasts respond to mechanical loading with increased expression of immediate early response genes and activation of multiple second messenger systems, which lead to mitosis and proliferation of cardiac fibroblasts and subsequent laying down of fibrillar collagen^{4,5, 6}. Numerous molecular pathways exist that could translate a mechanical signal to increased collagen production. Most notable among these are integrins and other cytoskeletal proteins that can transduce mechanical stretch into intracellular signaling pathways and subsequent synthesis of matrix proteins^{7,8}.

Connective tissue growth factor (CTGF), an immediate early response gene belonging to the CCN family, is a newly described mediator of collagen synthesis in various diseases⁹⁻¹¹. Recently, it has been demonstrated that CTGF is over-expressed in the dermal fibroblasts of scleroderma, a condition characterized by excessive collagen accumulation in the skin¹². Similarly, chronic infusion of ang II has been shown to increase CTGF production in both heart and blood vessels¹³. This latter finding indicated that the expression of CTGF may be regulated either by ang II, blood pressure or both. Recently, CTGF levels were shown to correlate with blood pressure in spontaneously hypertensive rats, suggesting that blood pressure may play a role in the regulation of CTGF expression¹⁴. In accordance with this, the expression of CTGF increased in response to high static pressure in renal mesangial cells¹⁵. However, how mechanical stimuli are related to CTGF production is not yet clear.

Therefore, we investigated whether mechanical stretch of cardiac fibroblasts enhances CTGF production, and explored the intermediary molecular mechanisms. We demonstrate a robust increase in CTGF expression in response to mechanical stretch, which is largely mediated by the EGF receptor via a β 1 integrin dependent pathway. We also show that this increase in CTGF can be efficiently blocked both in vitro and in vivo by treatment with tyrphostin, an inhibitor of EGF-R

activation. In vivo, treatment with tyrphostin was accompanied by decreased cardiac collagen content and myocardial CTGF levels. This provides new opportunities to target excess collagen accumulation in pathological forms of cardiac fibrosis.

Methods

Reagents

Dulbecco's modified eagle's medium (DMEM), fetal bovine serum (FBS), *L*-glutamate, penicillin, and streptomycin were purchased from GIBCO BRL, Breda, the Netherlands. Culture plates were obtained from Costar, Badhoevendorp, the Netherlands. Recombinant CTGF was synthesized as described earlier¹⁶. CTGF antibody, generated using *E.coli*-expressed mouse CTGF (a.a. 223-348) as an immunogen that reacts to mouse, human and rat CTGF, was purchased from Torrey Pines Biolabs, Inc, Houston, Texas, USA. Anti- β 1 integrin monoclonal antibody and a purified hamster anti-rat CD 29 (Ha 2/5) antibody (a β 1 integrin neutralizing antibody)¹⁷, which blocks binding of β 1 integrin to collagen⁸ was purchased from BD Biosciences Pharmingen, Leiden, the Netherlands. Anti-Phospho-P125FAK (P-P125FAK) antibody was from Cell Signaling Technology, Westberg BV, Leusden, the Netherlands. Tyrphostin (AG1478) and EGF were obtained from Sigma, Zwijndrecht, the Netherlands.

In vitro studies

Cardiac fibroblast culture

Cardiac fibroblasts were isolated from 2-day-old neonatal Sprague Dawley rats as described previously¹⁸. All the experiments were performed on cells from the second passage. Cells were maintained in DMEM supplemented with 10% FBS along with 1% *L*-glutamate, 50 U/mL penicillin and 0.1 g/L streptomycin, and were incubated at 37°C in a humidified chamber. Cells were placed in low-serum medium (0.1% FBS) for 24 hours before the experiment. Experiments were carried out according to the institutional guidelines for the experimental use of animals.

Stretching of cardiac fibroblasts

Cells were stretched using devices designed at the University of California, San Diego¹⁹. These devices apply a uniform, static and equibiaxial stretch to a silastic membrane (Specialty Manufacturing, Inc, USA) on which the cells were grown. It has previously been demonstrated that the fibroblasts receive the same deformation as the substrate¹⁹ and the amount of stretch applied to

the cells can be controlled by the user. A steady stretch of 10% was applied to the cardiac fibroblasts growing on to the silastic membrane. To reduce the hydrophobicity of the material, the membranes were routinely coated with type I collagen (0.01%, Sigma-Aldrich) in acetic acid (0.1M) for 3 hours at room temperature. After matrix coating, membranes and devices were sterilized by the ultraviolet irradiation.

Effects of CTGF on DNA and collagen synthesis

Cells were seeded into 6-well plates, grown to confluence and put to medium containing 0.01% FBS for 48 hours, and treated with recombinant CTGF for 18 hours. Synthesis of DNA and collagen was assessed by adding ^3H -thymidine and ^3H -proline for 24 and 48 hours, respectively. Subsequently, the cells were treated with 10% trichloroacetic acid to precipitate cellular DNA and protein that was later dissolved into 0.2% NaOH. The radioactivity was determined by counting the aliquot of each sample in a β -scintillation counter. An aliquot was used to determine total cellular protein. The incorporated ^3H -proline was corrected for cell density by dividing the radioactivity in counts per minute by the total amount of cellular proteins.

In vivo study

Study design

Hypertensive homozygous m(Ren2)27 transgenic rats were obtained from the Max-Delbrück-Zentrum für Molekulare Medizin, Berlin, Germany. Seven rats were treated with EGF-R blocker, Tyrphostin A46 (600 μg /Kg/day) and six with placebo for 4 weeks starting from the age of 7 weeks. Drugs were administered via osmotic minipumps (Alzet osmotic minipumps, Palo Alto, CA, USA; types 2ML4). Age matched wild type littermates were used as controls. Hemodynamic measurements were taken before sacrifice, and heart and lung weight were measured after the sacrifice. Experiments were carried out according to the institutional guidelines for the experimental use of animals.

Measurement of myocardial collagen content

Myocardial collagen was stained with picrosirius-red as described earlier²⁰. For the quantification of left ventricular collagen volume fraction (CVF), computerized planimetry was performed in 7 randomly selected fields per section per rat.

Protein measurement from cell lysate and myocardial homogenate

Protein isolation and western blotting were performed as described previously¹⁸. Total protein concentration was measured using the bicinchonic acid kit and equal amounts of the protein samples were loaded on the gel. Western blotting was performed with the specific antibodies against integrin $\beta 1$ (1/2000), P-P125FAK (1/1000) and CTGF (1:3000). To further ensure equal loading, the same membranes were probed with GAPDH (1:5000). Bands were visualized by enhanced chemiluminescence and quantified by a soft ware Quantity One (Bio Rad).

Statistical Analysis

The data are expressed as n-fold increase over control in densitometric arbitrary units as mean $\pm$ SEM. Data are derived from at least 3 different experiments performed in duplo. One-way ANOVA followed by Student-Newman-Keuls method was used for comparing the differences among multiple groups (Instat). Differences between the groups were considered to be significant when $P \leq 0.05$.

Results

Recombinant CTGF increased DNA and protein synthesis in cardiac fibroblasts

When synchronized cardiac fibroblasts were treated with 0-100ng/ml recombinant CTGF, DNA synthesis as assessed by ^3H -thymidine incorporation and protein synthesis as assessed by ^3H -proline incorporation were both stimulated in a dose-dependent fashion with maximal effect at 50ng/ml (Figure 1).

Figure 1. Effects of recombinant CTGF on DNA and collagen synthesis in cultured cardiac fibroblasts.

A. Effects of CTGF (10, 50, 200 ng/ml) on ^3H -thymidine incorporation in. B, cardiac fibroblasts were synchronized, treated with CTGF (10, 50, 200 ng/ml) and ^3H -proline was added for the final 48 hours followed by scintillation counting. Untreated cells were used as controls. *, $P < 0.05$ vs. controls, n = 8 per group.

β 1 integrin is essential for CTGF production in cardiac fibroblasts

Since β 1 integrin is shown to act as a mechanoreceptor in stretched cardiac fibroblasts⁸, we tested whether β 1 integrin signaling was important for the CTGF response to stretch. Stretched fibroblasts were treated with 10 μ g/ml of β 1 integrin neutralizing antibody 15 minutes prior to stretch. To ascertain whether the anti- β 1 integrin antibody blocked integrin signaling, we measured the major signaling molecule downstream of β 1 integrin, phosphorylated P125FAK in the same samples. P125FAK phosphorylation decreased significantly upon treatment by the neutralizing antibody. We also treated non-stretched cells with the β 1 integrin neutralizing antibody for 24 hours. In both stretched and non-stretched cells, β 1 integrin neutralizing antibody virtually abolished CTGF production (**Figure 3**). To exclude the non-specific effects of anti- β 1 integrin antibody, GAPDH levels were assessed in the same membranes, which did not reveal any effect of the β 1 integrin neutralizing antibody.

Figure 2. Time-course study of the effects of stretch on CTGF levels. Cardiac fibroblasts plated on collagen coated silastic membrane were stretched for a variable periods and CTGF levels were measured in the cell lysate. Upper panel: representative blot showing the effects of stretch on CTGF levels at different time points, lower panel: quantification of blots from 3 different experiments with controls set to unity. Controls, unstretched cells, *, $P < 0.01$ vs. controls.

EGF-R antagonist –tyrphostin, abolished CTGF production in cardiac fibroblasts

As β 1 integrin has been shown to activate EGF-R signaling²¹, we tested whether EGF-R activation was important for the CTGF response to stretch. Tyrphostin AG1478 is a small molecular weight synthetic compound that is reported to compete selectively with EGF-R for ATP, preventing the phosphorylation of EGF-R tyrosine residues²¹⁻²³. Cells were stretched for 3 hours either alone or in combination with a selective EGF-R blocker, tyrphostin AG1478 (10nM) and CTGF levels were measured in the cell lysate. CTGF increased 2.6 fold following stretch, which was completely abolished by tyrphostin. Similarly, in non-stretched cardiac fibroblasts grown on collagen-coated

plates, tyrphostin treatment (24 hours) reduced the basal level of CTGF (**Figure 4**). P-P125FAK levels remained unaltered after tyrphostin treatment of cardiac fibroblasts.

CTGF not increased by direct EGF application

Given the important role for the EGF-R, we addressed whether direct activation of the EGF-R by its endogenous ligand would also increase CTGF levels. Cardiac fibroblasts were treated with EGF (1 and 10 ng/ml) in both stretched and non-stretched conditions. EGF did not increase CTGF under basal conditions, nor did EGF enhance the response of CTGF to stretch (**Figure 5**). We also measured P-P125FAK levels in the cell lysate treated with EGF and found no difference in both basal as well as in stretched conditions (data not shown).

In vivo effects of tyrphostin on cardiac collagen and cardiac CTGF in (mRen2)27 rats

To investigate in vivo whether the EGF-R is important for cardiac CTGF regulation and cardiac collagen accumulation, we investigated homozygous hypertensive transgenic Ren2 rats that develop excess cardiac fibrosis and LV dysfunction by 13-15 weeks of age³. Myocardial CTGF levels in these rats were significantly increased compared to wild type controls. Treatment of a subgroup of (mRen2)27 rats with tyrphostin A46 significantly decreased LV end diastolic pressure (treated 5 ± 1.2 vs. placebo 13 ± 2.4 mmHg, $P < 0.05$), LV collagen content and myocardial CTGF levels compared to placebo-treated rats (**Figure 6**).

Figure 3. Effects of anti- $\beta 1$ integrin antibody on CTGF expression and P-P125FAK phosphorylation in cardiac fibroblasts. Cardiac fibroblasts were treated with anti- $\beta 1$ neutralizing antibody both under stretched (3 hours) and basal conditions and CTGF and P-P125FAK were measured in the cell lysate. **A**, upper panel: a representative blot showing the effects of anti- $\beta 1$ integrin antibody on CTGF levels, lower panel: quantification of blots. **B**, upper panel: a representative blot showing the effects of anti- $\beta 1$ integrin antibody on P125FAK phosphorylation, lower panel: quantification of blots. *, $P < 0.01$ vs. controls, $\beta 1$ -int ab, anti- $\beta 1$ integrin antibody.

Figure 4

Figure 5

Figure 4. Effects of tyrphostin on CTGF levels and P-P125FAK phosphorylation in cardiac fibroblasts under stretched (3 hours) and non-stretched conditions. **A**, bar diagrams representing the levels of CTGF in cardiac fibroblasts treated with and without tyrphostin compared to controls. Upper panel shows a representative blot that corresponds to the underlying bars. **B**, quantification of the levels of P-P125FAK in cardiac fibroblasts treated with and without tyrphostin compared to controls. Upper panel shows a representative blot that corresponds to the underlying bars. *, $P < 0.01$ vs. controls.

Figure 5. Effects of EGF on CTGF levels in cardiac fibroblasts. Synchronized cardiac fibroblasts were treated with and without (controls) EGF for 24 hours. The Bar diagram represents the level of CTGF detected by western blotting in the whole cell lysate. Upper panel shows a representative blot that corresponds to the underlying bars. *, $P < 0.01$ vs. controls.

Discussion

The main finding of this study is that CTGF robustly increases in response to mechanical stretch of cardiac fibroblasts and that this effect is to an important extent mediated by the epidermal growth factor receptor via a $\beta 1$ integrin dependent pathway. We show that this cascade can be efficiently blocked by tyrphostin leading to a decrease in cardiac collagen in vivo.

Effects and regulation of CTGF

CTGF is a member of CCN family proteins that induce cell adhesion, migration, proliferation and synthesis of collagen and other ECM proteins¹⁰. CTGF has been proposed to cause cardiac fibrosis since the cardiac expression of CTGF was found to be increased following myocardial infarction and hypertension, and fibroblasts were reported to increase collagen production in response to CTGF^{24,25 14}. Our findings that recombinant CTGF increases thymidine and proline incorporation confirm that CTGF indeed induces fibroblast proliferation and collagen synthesis in vitro. In agreement with this, *in-vivo* inhibition of CTGF production by tyrphostin was paralleled by a decrease in myocardial collagen content in transgenic (mRen2)27 rats.

It has been reported that growth factors like TGF $\beta 1$ and Ang II can induce CTGF expression both in vitro and in vivo¹³. A recent study suggested that CTGF expression may also be controlled by mechanical stimuli since the production of CTGF proportionately increased with increasing blood pressure in spontaneously hypertensive rats¹⁴. Similarly, increased levels of myocardial CTGF were reported in renovascular hypertension¹¹. Although these reports indicate that mechanical stretch might increase CTGF production, the possibility that CTGF was regulated by the increase levels of ang II or TGF $\beta 1$ are still unanswered since ang II and TGF $\beta 1$ are known to be upregulated in the animal models employed in these studies. Therefore, direct evidence demonstrating that mechanical stimuli cause an increase in CTGF production has so far been lacking. Our finding that CTGF levels increased 2.6 fold following static stretch of cardiac fibroblasts directly supports the notion that CTGF levels can also be modulated by mechanical stimuli. Since CTGF levels were increased as early as 3 hours following mechanical stretch, increase in CTGF expression secondary to de novo production of ang II following stretch is unlikely. Moreover, we noted that neutralizing antibody against $\beta 1$ integrin receptor abolished the increase in CTGF in response to stretch indicating a direct effect of stretch for CTGF production.

Figure 6. Effects of tyrphostin on LV collagen volume fraction (CVF) and CTGF levels. **A**, quantification of left ventricular interstitial CVF in wild type, tyrphostin treated and untreated Ren2 rats. Upper panel shows the representative photomicrograph of left ventricular CVF. **B**, Bar diagram representing the myocardial CTGF levels normalized to GAPDH in tyrphostin treated and untreated rats. Upper panel corresponds to the representative blot of the underlying bar. Ren2plac, placebo-treated (mRen2)27 rats, Ren2tyr, tyrphostin treated (mRen2)27 rats, WT, wild type controls, *, $P < 0.05$ vs. wild type.

Stretch, integrins and CTGF

Integrins are cell surface adhesive receptors formed by α and β subunits, which bind to extracellular matrix proteins like collagen and fibronectin. Integrins associate with signaling molecules in the focal adhesion complex, which act both as a signaling device and a connection to cytoskeleton. Therefore, integrins are considered as the sensor and transducer of mechanical stimuli in the cell²⁶. Stretching cardiac fibroblasts increased β 1 integrin expression, which was accompanied by increased CTGF production. A neutralizing anti- β 1 integrin antibody abolished the CTGF increase suggesting that signaling through β 1 integrin is essential for stretch induced CTGF production. We also noticed a reduction of CTGF following the addition of anti- β 1 antibody at the basal state. This could be due to the fact that also in quiescent culture conditions, signaling through the β 1-integrin is not fully silenced since β 1 integrin is necessary for the adhesion of cardiac fibroblasts to each other and to the extracellular matrix. Therefore, β 1 integrin function seems crucial for CTGF regulation, both under basal and under stimulated conditions. Since anti- β 1 antibody blocks the adhesion of cells selectively to collagen⁸, this suggests that adhesion of cardiac fibroblasts to collagen might positively regulate CTGF production.

Integrin, EGF-R and CTGF

Upon stimulation, integrins interact with transducing molecules like P125FAK, whose aminoterminal domain binds to the intracellular domain of the $\beta 1$ and $\beta 3$ integrins while its carboxyterminal binds to SH2 and SH3 domain of several proteins involved in focal adhesion assembly and signal transduction²⁷. Upon activation, P125FAK phosphorylates at its tyrosine residues and initiates signal transduction. Apart from P125 FAK, integrins can also activate c-Src, Shc and phosphotidylinositol 3-kinase²⁸. Moreover, integrins also interact and synergize with various growth factor receptors to initiate specialized signalling responses, and that depend on the particular subunit of integrins²³. Recently, Moro and colleagues have shown that $\beta 1$ integrin activates EGF-R tyrosine kinase in response to cell adhesion and that this process is independent of P125 FAK phosphorylation. Phosphorylation of EGF-R by $\beta 1$ integrin involves its tyrosine residues at positions 845, 1068, 1086 and 1173 leading to Shc phosphorylation and MAPK activation. In contrast, EGF stimulation phosphorylates the EGF-R at a different tyrosine residue, 1148²¹.

An earlier report has shown that $\beta 1$ integrin activates EGF-R in primary human fibroblasts under adherent culture condition²³. We observed a discernible amount of CTGF even under quiescent culture conditions in cardiac fibroblast. This could be due to the fact that $\beta 1$ integrin coupled activation of EGF-R stimulates CTGF production in adherent cardiac fibroblasts as the addition of tyrphostin in the culture medium reduced the levels of CTGF in both stretched and non-stretched cells. Similarly, tyrphostin treatment significantly reduced CTGF and collagen levels in the myocardium of (mRen2)27 rats. It is intriguing that while tyrphostin AG1478, which non-selectively blocks the phosphorylation of different tyrosine residues on EGF-R, inhibited stretch mediated CTGF response, the classic ligand, EGF itself, was unable to induce CTGF. This latter finding is in agreement with a recent publication by the group of Tamatani who showed that CTGF production was not affected by EGF treatment of the cultured human fibroblasts²⁹. Although it is not yet clear whether phosphorylation of different tyrosine residue of EGF-R leads to the activation of distinct cellular pathways, our findings lead us to speculate that CTGF in fibroblasts is controlled by $\beta 1$ integrin mediated phosphorylation of the EGF-R at residues 845, 1068, 1086, and 1173 and that the dominant phosphorylation site of EGF, tyrosine residue 1148, possibly does not control the CTGF response to stretch or adhesion.

Study implications

The main therapeutic implication of our results is that production of CTGF and subsequent induction of collagen accumulation may be counteracted by a specific inhibition of β 1-integrin coupled EGF-R activity, so that EGF-R antagonists, like tyrphostin would be effective. However, inhibition of EGF production itself is less likely to yield any effect on CTGF expression and collagen deposition. Alternatively, selective blockade of signalling through the integrin β 1 subunit may also abolish the production of CTGF leaving the other functions mediated by EGF largely unaffected.

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Schematic presentation of the main findings

8

General Discussion

Hypertrophy and fibrosis of the myocardium is the major mechanism underlying disability and death due to diseases such as hypertension and myocardial infarction (MI)¹. It is a complex process that involves changes in structure, shape and topography at the global level and changes in myocytes and non-myocytes at cellular and sub-cellular levels that negatively affects cardiac performance². Multiple lines of evidence demonstrate that the cardiac RAS is linked to pressure-overload remodeling since the local RAS has been shown to be activated in experimental LVH induced by hemodynamic overload³⁻⁶. Notwithstanding the high efficacy of current treatment, cardiac hypertrophy and particularly fibrosis propagate to lead to cardiac dysfunction or dysrhythmias. The lack of effective treatment to prevent or reverse cardiac fibrosis exemplifies the inadequate understanding of this process and hence the lack of optimal attempts to prevent it. The two major questions are- firstly, how increased loading and stretch is transduced to an increase in collagen deposition and secondly, how activated neurohormonal systems, most remarkably the RAS, translates to increased fibrosis.

With regard to the molecular mechanisms by which external overload evokes cardiac hypertrophy and fibrosis, activation of second messengers such as Raf kinase, MAP kinase and Smads have been implicated^{2,7}. This thesis focuses on the identification and characterization of signal transduction pathways that participate in RAS-mediated cardiac remodeling during heart failure. Our studies elucidate that TGF β 1 signaling acts as a major regulatory determinant of RAS-induced cardiac dysfunction.

Ang II, TGF β and myocardial remodeling

The majority of the known cardiovascular activities of Ang II are mediated through the binding of Ang II to its high-affinity AT₁ receptors. Stimulation of the AT₁ receptors may trigger a variety of different tyrosine kinases and stimulate the early gene response that control cardiac matrix growth and hypertrophy^{2,7}. A signal transduction event stimulated by Ang II include the activation of phospholipase C, inositol triphosphate formation, activation of protein kinase C, and protein tyrosine phosphorylation⁸⁻¹². Activation of MAP kinases serves as the downstream signalling mediator of aforementioned protein tyrosine cascades. In **chapter 3**, we discussed the role of ERK signalling in transgenic rat model over-expressing renin leading to high Ang II (Ren2 rats) and demonstrated that inhibition of the phosphorylation (activation) of ERK leads to reduction of LVH and myocardial collagen deposition and improves cardiac function.

In addition to Ang II, TGF β 1 is known to participate in matricellular growth associated with cardiac hypertrophy and heart failure. Expression of TGF β 1 is increased in the cardiac hypertrophy and fibrosis resulting from MI and pressure overload¹³⁻¹⁵. Consistent with the model that Ang II and TGF β are functionally linked, Ang II blockade reversed myocardial fibrosis and expression of TGF β in tandem¹⁶, whereas blocking antibodies to TGF β inhibited myocyte hypertrophy caused by the conditioned medium of Ang II-treated cells¹⁷. Effects of TGF β 1 are mediated by activation of serine-threonine residues of trans-membrane receptors, TGF β receptor type I and type II¹⁸. This leads to the phosphorylation and activation of downstream signalling molecules including MAPK (ERK, P38, JNK) and Smad2/3 that transmit the TGF β 1 signal from the cytoplasm to the nucleus¹⁸. It has been shown that chronic blockade of AT1 receptors leads to the inhibition of TGF β 1 synthesis and Smad phosphorylation leading to a decrease in cardiac collagen content¹⁶. In line with these reports, we found that Smad2 phosphorylation was increased in Ang II over-expressing Ren2 rats and that an AT1 blocker inhibited P-Smad2 and reversed cardiac fibrosis (**chapter 3**). Moreover, an AT1 blocker also prevented the development of LVH in these transgenic animals, which could be mediated by lowering of TGF β itself.

Previous studies have addressed the function of TGF β 1 in the myocardium. Mice somatically lacking the TGF β 1 gene were susceptible to fatal immune reactions and these mice were kept under strict aseptic conditions. In these mice, Schultz and colleagues demonstrated that chronic treatment with sub-pressor doses of Ang II failed to induce LVH and ventricular dysfunction but there was no significant difference in myocardial fibrosis between Ang II treated wild type and TGF β 1 null mice¹⁹. However, strict aseptic housing and the use of very low dose of Ang II make it difficult to draw definite conclusions. In another study, chronic infusion of a neutralizing antibody against TGF β 1 prevented pressure overload induced myocardial collagen deposition and improved diastolic cardiac function while cardiac hypertrophy was largely unaltered²⁰. However, TGF β receptors exist in both myocytes and non-myocytes. From these two reports, it is still impossible to draw conclusions regarding the role of TGF β signalling in myocytes. What had been missing until now was a *bona fide* genetic study in mice that would circumvent the problem imposed by lethality of somatic inactivation of TGF β 1. Therefore, we generated mice where we were able to conditionally inactivate the TGF β 1 type I receptor by inducing *Cre-recombinase* coupled to myocyte specific promoter that was dependent on tamoxifen for its activation. This allowed eliminating TGF β 1 signaling selectively in the cardiomyocytes cells at a given moment of postnatal life. We induced

cardiac hypertrophy and dysfunction by chronic infusion of Ang II in these mice. This revealed that a post-natal loss of the TGF β type 1 receptor selectively in cardiomyocyte fully protected the heart against hypertrophy and dysfunction caused by a 4-week Ang II infusion. Immunohistochemical analysis and subsequent western blotting showed that this effect was related to lack of activation of P38 MAPK, localized predominantly in the cardiomyocytes and was not related to altered SMAD signaling (**chapter 6**). This conditional knock out approach provided the first firm and direct evidence that loss of TGF β signaling in cardiomyocytes completely interrupts Ang II-induced LVH and cardiac dysfunction which we showed to be due to the loss of TGF β -coupled P38 activation rather than Smad activation. *Collectively, it suggests that TGF β type I receptor-coupled P38 signaling in myocytes plays a dominant role in Ang II-induced cardiac hypertrophy and dysfunction.*

ACE, AcSDKP, Smad and fibrosis

Apart from Ang II, the Ang II generating enzyme, ACE, serves multiple functions that go vastly beyond its role in the RAS. The importance of ACE in regulating cardiovascular biology has been implicated by the findings that marked induction of ACE occurs in almost all models of cardiac injury including pressure and volume overload and myocardial infarction leading to heart failure²¹. Upregulation of cardiac ACE is particularly shown to be associated with cardiac fibrosis since fibrotic areas of the heart display the highest levels of ACE activity^{22,23}. Apart from Ang I, ACE can metabolize and inactivate AcSDKP, a tetra-peptide known to inhibit the growth of hematopoietic stem cells²⁴. Based on the assumption that structural homogeneity of normal adult myocardium is governed by a balanced equilibrium between stimulators and inhibitors of cell growth, we proposed that AcSDKP could act as a functional antagonist of Ang II. To address our hypothesis, we performed in vitro experiments, which showed that AcSDKP inhibits the progression of cell cycle of cardiac fibroblasts. In line with this finding, Rhaleb and colleagues showed that AcSDKP was able to inhibit collagen synthesis both in vitro and in vivo without any effects on blood pressure^{25,26}. Given the powerful effects of TGF β 1 on cardiac fibroblasts proliferation and collagen deposition, we investigated whether AcSDKP could modify TGF β 1 post-receptor signalling. The data showed that AcSDKP inhibits the phosphorylation and nuclear translocation of Smad2 in cardiac fibroblasts (**chapter 4**).

Although increased levels of ACE had been associated with cardiac hypertrophy and fibrosis, no study to date had shown the direct cause effect relationship between ACE and myocardial

morphology. In order to answer this question, we studied transgenic rats over-expressing high levels of ACE selectively in the cardiomyocytes and studied their phenotype (**chapter 5**). These rats developed LV interstitial fibrosis, which was accompanied by a decrease in AcSDKP levels depending on the ACE gene-dose while cardiac Ang II level was not elevated. In line with the fact that AcSDKP inhibits Smad phosphorylation, levels of phosphorylated Smad2/3 in the myocardium of ACE-rats were increased. Treatment of these rats with an ACE-inhibitor normalized the cardiac AcSDKP concentration, Smad2/3 phosphorylation and decrease in cardiac collagen content. Intriguingly, ACE-transgenic rats did not develop myocardial hypertrophy. *Taken together, Smad signalling appears to play a central role in ACE induced cardiac fibrosis, but does not seem to be related to myocyte hypertrophy.*

Mechanical stretch, CTGF and collagen deposition

Mechanical stretch per se is a major factor for cardiac hypertrophy due to pressure or volume overload leading to cardiac restructuring and remodeling²⁷. A series of studies have shown that mechanical stretch is able to induce the expression of specific genes that cause myocyte hypertrophy, cardiac fibroblasts proliferation and collagen synthesis either directly or secondary to the recruitment of neurohumoral factors²⁸⁻³¹. Apart from TGF β 1 and Ang II, CTGF is a recently discovered and potent molecule that promotes multi-organ fibrogenesis^{28,29,32,33}. CTGF expression is regulated by several agents including TGF β 1 and Ang II^{28,34,35}. A recent study suggested that CTGF expression may also be controlled by mechanical stimuli as a positive correlation was shown between CTGF levels and blood pressure in spontaneously hypertensive rats³¹. Increased levels of myocardial CTGF were also reported in a rat model of renovascular hypertension³⁶. In vitro, the expression of CTGF increased in response to high static pressure in renal mesangial cells³⁰. However, whether mechanical stretch regulates CTGF expression in cardiac fibroblasts was not known. More importantly, the cellular signaling linking mechanical stimuli to CTGF production was not yet established. Therefore, we investigated whether mechanical stretch of cardiac fibroblasts enhances CTGF production, and explored the intermediary molecular mechanisms (**chapter 7**). We demonstrated that mechanical stretch induces CTGF expression via an EGF-receptor coupled β 1 integrin signaling pathway and the increased CTGF response was efficiently blocked by treatment with an EGF-R inhibitor-tyrphostin, both in vitro and in vivo. It is known that Ang II and TGF β 1 are upregulated in response to mechanical stretch, and that both these molecules induce CTGF production. Since CTGF levels were increased as early as 3 hours following mechanical stretch, early increase in CTGF expression secondary to de novo production of Ang II

and TGF β 1 following stretch is unlikely. This led us to conclude that mechanical stretch is able to initiate fibrotic response by increasing the expression of fibrotic mediators like CTGF. This response is perpetuated in the later stage by up-regulation of Ang II and TGF β 1. *In conclusion, CTGF is the primary mediator of stretch induced cardiac collagen synthesis.*

Conclusion and future directions

In conclusion, the results presented in this thesis indicate that RAS-induced cardiac matrix regulation depends on the balanced equilibrium between its inhibitory (AcSDKP) and stimulatory (Ang II) players. Both of these molecules seem to act on the same effectors (Smad2/3) either to facilitate or inhibit their activities. Ang II, the primary agent that stimulates matrix deposition, evokes multiple signaling pathways that are linked to its G-protein coupled receptor. TGF β signaling appears to act as the crucial mediators in this regard. We noted that signaling through TGF β regulates myocyte hypertrophy via P38 signaling while activation of Smad signaling predominantly regulates the fibrotic response. On the other hand, AcSDKP inhibits the matricellular response via inhibition of the Smad signaling cascade. Identification of these molecular mechanisms opens up the possibility to design a therapy that selectively intervenes Smad or P38 signaling so that either fibrosis or hypertrophy can be selectively prevented. To further distinguish the effects of TGF β signaling in myocyte versus fibroblasts, a comparative study needs to be designed employing an additional model where TGF β signaling is disrupted selectively in cardiac fibroblasts. This necessitates a generation of mice expressing inducible *Cre recombinase* under the control of fibroblast-specific promoter. Then a similar strategy by which myocyte-specific TGF β receptor disruption was performed (chapter 6) can be applied to delete TGF β receptor signaling in cardiac fibroblasts.

Similarly, we have observed that mechanical stretch induces CTGF, a strong fibrotic stimulus, via β 1-integrin-coupled EGF-receptor signaling. In vivo, blockade of EGF-receptor activity decreased CTGF expression and prevented the deposition of collagen in the myocardium in Ang II-induced hypertensive (mRen2)²⁷ transgenic rats. This indicates that CTGF acts as the primary mediator of hypertensive cardiomyopathy that necessitates EGF-receptor signalling. To confirm whether CTGF is a sole regulator of matrix-response in hypertensive hearts, a loss of function study should be designed. The simplest and direct way to address this issue would be to induce hypertension in animals lacking CTGF gene and study their cardiac phenotype. These studies will facilitate designing a therapeutic agent that selectively blocks over-expression of CTGF so that cardiac

fibrosis secondary to hypertension can be counteracted. Since CTGF is a secretory protein, measurement of CTGF levels in the serum of hypertensive patients and its correlation to cardiac functions would illuminate whether or not CTGF can be used as a marker of hypertensive heart disease.

Finally, LV hypertrophy and fibrosis has currently been a subject of intensive research so that optimal therapy can be designed to counteract these adverse changes. At present, drugs like ACE-inhibitors and AT1 blockers are routinely used. However, these medicines are only partially able to antagonize the adverse effects imposed by an activated RAS. The development of additional therapeutic agents that more effectively antagonize the critical cellular signalling effectors such as Smad, P38 and the EGF-receptor may yield better outcomes.

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Figure 1 depicts the major findings presented in this thesis. High levels ACE leads to a decrease in the levels of AcSDKP while the levels of cardiac Ang II does not directly depend on the activity of ACE. High levels of Ang II increase blood pressure and activate TGFβ, which initiates its signalling system. Signaling through TGFβ is conveyed by activated downstream effectors like Smad and P38. Activation of P38 leads to myocardial hypertrophy while Smad2/3 phosphorylation induces a fibrotic response. High blood pressure and stretch, on the other hand, increases the expression of CTGF that also induces a matricellular response. In contrast, AcSDKP inhibits Smad2/3 phosphorylation leading to inhibition of fibrosis. Excess degradation of AcSDKP by increased levels of ACE can, therefore, lead to an exaggerated fibrotic response.

Summary

Introduction

Hypertensive hearts adapt to an increased workload by cardiac increasing the size of myocytes and altering the levels and composition of matrix. However, these initial adaptive changes can ultimately contribute to cardiac dysfunction, which is often characterized by excess cardiac collagen deposition. It is probably extravagant that pressure-overloaded ventricles, where myocytes are growing to cope with the increased workload, generate excess collagen. Excess collagen renders the ventricles stiffer and heterogeneous, thus increasing the propensity to develop arrhythmias. So the question arises why and under what mechanisms the excess collagen is formed in a pressure loaded heart. Answers to these questions may help to design a rational way to prevent or inhibit adverse accumulation of collagen in the heart. A central concept is that activation of the rennin angiotensin system (RAS) is a universal accompaniment to cardiac fibrosis and ventricular dysfunction. This thesis was based on the hypothesis that an activated RAS initiates signaling events in cardiac fibroblasts that leads to increased cardiac collagen, and that interruption of this signaling can *selectively* halt collagen formation and/or hypertrophy. This hypothesis predicts that selective signaling cascades exist that regulate either myocyte hypertrophy or collagen synthesis, so that a particular pathway can be targeted to specifically inhibit hypertrophy or fibrosis.

Chapter 1 comprises the **general introduction** to the thesis and the historical remarks on RAS and TGF β 1 system. This provides an overview on the impact of cardiac fibrosis on ventricular function, role of RAS and the contribution from its stimulatory (e.g. Ang II) and inhibitory (e.g. AcSDKP) players on cardiac remodeling. It further underscores the effects of TGF β 1 signaling system on cardiac fibrosis.

Chapter 2 gives a brief summary of the **molecules that are involved in cardiac hypertrophy** and focuses on factors that lead to transition from compensatory hypertrophy to maladaptive state. Based on the existing body of literature, role of TGF β 1/Smad signaling is highlighted.

In Chapter 3, we studied the **molecular changes resulting from high cardiac Ang II**. The Homozygous variant of the transgenic rats over-expressing renin TGR(mRen2)27 had hypertension, LV hypertrophy, excessive myocardial fibrosis and impaired LV function. At the second messenger level, both Smad2 and ERK 42/44 were activated. Inhibition of either Smad2 or ERK

phosphorylation by losartan and tyrphostin, respectively, abolished myocardial remodeling and improved LV performance. This suggests that cross-talk and co-ordinated action between these signaling pathways is mandatory for Ang II-induced ventricular remodeling and blockade of either of the signaling arms is sufficient to prevent the adverse effects.

AcSDKP is a novel peptide linked to the RAS and cellular growth. When the work for this thesis was initiated, very little was known about the function of AcSDKP in cardiac biology. The only known function of AcSDKP was that it is a negative regulator of the growth of hematopoietic stem cell progenitors. We presumed that a reciprocal regulation normally exists between the growth stimulators and inhibitors to ensure a balanced equilibrium to maintain the normal steady state in organ structure. A relative excess of stimulators, due to a paucity of inhibitors, creates a state favorable to tissue growth, which, if persistent, may lead to pathologic structural remodeling of the heart. Since AcSDKP, a peptide that depends exclusively on ACE for its degradation, inhibits hematopoietic cells proliferation, we hypothesized that AcSDKP is a negative regulator of cardiac fibrosis.

In Chapter 4, we studied the effects of AcSDKP on the cell-cycle of cardiac fibroblasts. We established that AcSDKP exerts its effects by inhibiting phosphorylation and nuclear translocation of Smad2. **In chapter 5, we established the importance of AcSDKP in counteracting fibrosis induced by ACE.** Generating transgenic rats over-expressing ACE selectively in the heart, we studied their phenotype with and without ACE inhibitor, lisinopril. We demonstrated that levels of cardiac AcSDKP correlated inversely with the ACE expression profile and that a reciprocal relationship exists between the levels of AcSDKP and cardiac collagen content. In search of the underlying mechanism that could explain this phenotype, we found that phosphorylation of Smad2 and Smad3 was increased in ACE-transgenic rats depending on the ACE-gene dose. Despite increased phosphorylation of Smad2/3, TGF β 1 mRNA was not upregulated in ACE-transgenic rats. Competition ligand binding assay demonstrated that AcSDKP does not compete with TGF β 1 for its receptor binding. In cultured cardiac fibroblasts, AcSDKP was able to inhibit TGF β 1 induced Smad3 phosphorylation within 60 minutes, which indicated that AcSDKP inhibits Smad phosphorylation directly at intracellular level.

Having identified TGF β 1/Smad signaling cascade as a central mediator of cardiac remodeling, we hypothesized that targeted deletion of TGF β 1 signaling in myocardium renders hearts resistant to remodeling induced by Ang II. In **chapter 6**, we generated mice where a conditional Cre/LoxP system would be induced by tamoxifen to inactivate the gene for TGF β 1 type I receptors specifically in cardiac myocytes. The system was induced in the mice older than 10-weeks so that the embryonic or early postnatal effects of the loss of cardiac TGF β 1 type I receptors would be circumvented. This showed that **mice deficient of TGF β 1 type I receptors specifically in cardiomyocytes were resistant to Ang II induced cardiac hypertrophy and dysfunction**. This was accompanied by failure of P38 MAPK activation, while SMAD signaling remained unchanged. We concluded that TGF β receptor-coupled P38 activation in cardiomyocytes is essential for Ang II-induced cardiac hypertrophy.

Although long standing hypertension or pressure overload can lead to activation of RAS, it was not clear whether mechanical stretch alone is able to trigger a signal to initiate fibrosis. In **chapter 7**, we utilized an equi-biaxial stretch system and studied the **effects of passive stretch on cardiac fibroblasts** grown in collagen coated silastic membranes. We demonstrated that mechanical stretch induced the release of a potent fibrotic mediator, CTGF, as early as 3 hours following stretch stimulus via EGF-receptor coupled β 1 integrin signaling.

In conclusion, this thesis unravels how RAS activation is translated to untoward cardiac effects, and demonstrates an unsuspected and novel mechanism of action for the long known enzyme ACE. Many effects of Ang II are mediated via TGF β system, where the effects on fibroblasts are due to recruitment of Smads, while we show direct evidence that the effects in myocytes are primarily due to recruitment of P38 MAPK. Surprisingly, ACE again plays a major role in this system, not by its Ang II generating capacity, but rather by its breakdown of AcSDKP, which we show to be an important inhibitor of Smad signaling, so that an increase in ACE results in the loss of this beneficial inhibitor. Since these signaling cascades have been previously described to be active in various types of cancer, the recent development of drugs intervening in these cascades opens novel possibilities beyond cancer treatment, so that the knowledge gathered above can help to choose the most appropriate of these newly developed inhibitors to target cardiac hypertrophy, fibrosis or both.

Samenvatting

Inleiding

Een hart dat blootgesteld wordt aan hoge bloeddruk, past zich aan deze verhoogde belasting aan door vergroting van de hartspiercellen (hypertrofie) en door het veranderen van de samenstelling van de extra-cellulaire matrix. Deze initiële nuttige aanpassingen zullen echter uiteindelijk leiden tot disfunctioneren van het hart, wat dikwijls gepaard gaat met een overmatige collageen depositie in het hart. De hoeveelheid collageen die de ventrikels produceren in reactie op druk-overbelasting, waarbij de hartspiercellen groeien om deze verhoogde belasting aan te kunnen, is waarschijnlijk te overvloedig. Deze enorme hoeveelheid collageen maakt de ventrikels stijver en door de heterogene verdeling ervan wordt de kans op arrhythmieën verhoogd. De belangrijkste vragen zijn dus waarom en door welk mechanisme deze enorme hoeveelheid collageen wordt gevormd in reactie op druk-overbelasting van het hart. De antwoorden op deze vragen zouden kunnen helpen om op een rationele manier de ongewenste opstapeling van collageen in het hart te voorkomen of te blokkeren. Centraal concept hierbij is dat het renine angiotensine systeem (RAS) altijd geactiveerd wordt tijdens de vorming van cardiale fibrose en tijdens het optreden van ventrikel disfunctie. In deze dissertatie staat de hypothese centraal dat activatie van het RAS signalering initieert in cardiale fibroblasten leidend tot verhoogde hoeveelheden collageen in het hart en dat onderbreking van deze signalen *selectief* een halt kan toeroepen aan de collageenvorming en/of hypertrofie. Deze hypothese voorspelt dat er selectieve signaalcascades bestaan die of de hartspiercelgroei of de collageen synthese reguleren, zodat deze cascades als doelwit kunnen dienen om specifiek de hypertrofie of fibrose tegen te gaan.

Hoofdstuk 1 geeft een algemene inleiding voor deze dissertatie, waarin enkele historische feiten over het RAS en TGF β 1 systeem worden vermeld. Hierin wordt een overzicht gegeven van de impact van cardiale fibrose op ventrikel functie en over de rol van RAS, en de bijdrage van stimulatie (bv. d.m.v. Ang II) en inhibitie (bv. d.m.v. AcSDKP) op remodelering van het hart. Tevens wordt het belang van de effecten van het TGF β 1 signaalsysteem op cardiale fibrose beschreven.

Hoofdstuk 2 geeft een korte samenvatting van de moleculen die betrokken zijn bij cardiale hypertrofie, en richt zich met name op de factoren die betrokken zijn bij de overgang van

gecompenseerde hypertrofie naar decompensatie en hartfalen. Gebaseerd op bestaande literatuur wordt de rol van TGF β 1/Smad signalering toegelicht.

In hoofdstuk 3 worden de moleculaire effecten van verhoogd cardiaal Ang II bestudeerd. De homozygote variant van de transgene rat TGR(mRen2)27, die het renine 2 gen tot overexpressie brengt, ontwikkelde hypertensie, linker ventrikel hypertrofie, overmatige myocardfibrose en linker ventrikel disfunctie leidend tot hartfalen. Op het second messenger niveau waren zowel Smad2 als ERK 42/44 geactiveerd. Inhibitie van fosforylatie van Smad2 of ERK door respectievelijk losartan of tyrfostin voorkwam myocardiale remodelering en verbeterde de linker ventrikel functie. Dit suggereert dat crosstalk en gecoördineerde samenwerking tussen deze twee signaalcascades noodzakelijk is voor Ang II geïnduceerde ventriculaire remodelering. Blokkering van één van deze signaalcascades is voldoende om de ongunstige effecten te voorkomen.

AcSDKP is een nieuw peptide dat een rol speelt in RAS en bij cellulaire groei. Bij de start van dit onderzoek was er weinig gekend over de functie van AcSDKP in het hart. De enige bekende eigenschap van AcSDKP was dat het een negatieve regulator was van de groei van hematopoïetische stamcellen. Wij veronderstelden dat er gewoonlijk een wederzijdse regulatie bestaat tussen groei stimulators en remmers om een uitgebalanceerd evenwicht te verzekeren waardoor de normale steady-state toestand in orgaanstructuren wordt gehandhaafd. Een relatief surplus van stimulators, door een gebrek aan remmers, creëert een ideale conditie voor weefselgroei welke, indien continu aanwezig, kan leiden tot pathologische structurele remodelering van het hart. Omdat AcSDKP, een peptide dat uitsluitend gemetaboliseerd wordt door ACE, de hematopoïetische celproliferatie inhibeert, is onze hypothese dat AcSDKP een negatieve regulator is van cardiale fibrose.

In hoofdstuk 4 werden de effecten van AcSDKP op de celcyclus van cardiale fibroblasten bestudeerd. Wij toonden aan dat AcSDKP werkt door middel van remming van de fosforylatie en het nucleair transport van Smad2. **In hoofdstuk 5** bewezen we het belang van AcSDKP om ACE geïnduceerde fibrose tegen te gaan. We genereerden transgene ratten die ACE selectief in het hart tot overexpressie brengen, en bestudeerden hun fenotype in aan- of afwezigheid van de ACE-remmer lisinopril. Wij toonden aan dat de AcSDKP spiegels *invers gecorreleerd* zijn met ACE expressie en dat er een wederzijdse relatie bestaat tussen de AcSDKP concentratie en cardiale hoeveelheid collageen. Op zoek naar het onderliggende mechanisme dat dit fenotype kon verklaren,

vonden we dat de fosforylatie van Smad2 en Smad3 toegenomen was in ACE transgene ratten waarbij de stijging afhankelijk was van de ACE-gen dosis. Ondanks de verhoogde fosforylatie van Smad2/3 was het TGF β 1 mRNA niet upgereguleerd in ACE transgene ratten. Een competitie-ligand bindingsstudie toonde aan dat AcSDKP niet in competitie treedt met TGF β 1 voor de binding van TGF β 1 aan zijn receptor. In gekweekte cardiale fibroblasten was AcSDKP in staat om TGF β 1 geïnduceerde fosforylatie van Smad3 te inhiberen binnen één uur, wat aangaf dat AcSDKP op een directe manier de Smad fosforylatie inhibeert op een intracellulair niveau.

De identificatie van de TGF β 1/Smad signaalcascade als centrale mediator van cardiale remodelering, bracht ons tot de hypothese dat onderbreking van het TGF β 1 signaal specifiek in de cardiomyocyten het hart resistent maakt tegen Ang II geïnduceerde remodelering. **In hoofdstuk 6** genereerden we muizen die na inductie met Tamoxifen door een conditioneel Cre/LoxP systeem het gen voor de TGF β 1 type I receptor inactiveerden, specifiek in de hartspiercellen. Dit systeem werd geïnduceerd in muizen ouder dan 10 weken om zo de embryonale of vroeg postnatale effecten die door het verlies van de TGF β 1 type I receptor in het hart zouden kunnen optreden te omzeilen. Wij vonden dat muizen die de TGF β 1 type I receptor specifiek missen in cardiomyocyten, resistent waren tegen Ang II geïnduceerde cardiale hypertrofie en disfunctie. Dit ging samen met het falen van de p38 MAPK activatie, terwijl de Smad signaaltransductie onveranderd bleef. Wij concludeerden dat de TGF β receptor gekoppelde p38 activatie in cardiomyocyten essentieel is voor Ang II geïnduceerde cardiale hypertrofie.

Ondanks dat hypertensie of druk-overbelasting kan leiden tot de activatie van RAS, was het nog niet duidelijk of mechanische stretch alleen voldoende is om een signaal te initiëren dat leidt tot fibrose. **In hoofdstuk 7** gebruikten we een equi-bi-axiaal stretch systeem en bestudeerden de effecten van passieve stretch op cardiale fibroblasten, die in met collageen gecoate siliconenmembranen opgegroeid waren. Wij toonden aan dat mechanische stretch de excretie van de potente fibrotische mediator CTGF induceerde, reeds na 3 uur van de stretch stimulus, via EGF-receptor gekoppelde β 1 integrin signalering.

Concluderend ontrafelt deze dissertatie hoe RAS activatie vertaald wordt naar cardiale effecten en toont ze een onverwachte, nieuwe werking en mechanisme van het lang gekende ACE enzym. Veel effecten van Ang II worden gemedieerd door het TGF β systeem. In fibroblasten worden de effecten

van Ang II gemedieerd via TGF β afhankelijke Smad activatie. In cardiomyocyten daarentegen tonen wij aan dat de effecten van Ang II voornamelijk het gevolg zijn van activatie van p38 MAPK. Tot onze verrassing bleek ACE ook in dit systeem een belangrijke rol te spelen, echter niet door de Ang II generende eigenschap, maar eerder door afbraak van AcSDKP. Wij tonen aan dat AcSDKP een belangrijke remmer is van Smad activatie, zodat een stijging in ACE resulteert in een afname van deze heilzame remmer. Omdat eerder beschreven is dat deze signaalcascades geactiveerd worden bij verschillende vormen van kanker, opent de recente ontwikkeling vangeneesmiddellen die ingrijpen in deze cascades nieuwe mogelijkheden voor behandeling die verder strekken dan de behandeling van kanker alleen. Hierdoor zal de door deze dissertatie opgeleverde kennis van de signaalcascades in het hart helpen om de juiste van deze nieuwe ontwikkelde remmers te kiezen om cardiale hypertrofie, fibrose of beiden tegen te gaan.

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They say it takes a minute to find special persons, an hour to appreciate them, a day to love them, but then an entire life to forget them.

Curriculum vitae

Saraswati Pokharel was born on July 20th 1972 in Jogimara, Nepal. After completing the high school education from Nepal Secondary School, Tandi, Chiawan in 1989, she enrolled to Tribhuvan University, Institute of Medicine for Intermediate of Health Science studies. In 1992, she briefly worked for United Mission to Nepal, Amp-Pipal Hospital, Gorkha as a paramedic. Shortly after that she was chosen to start medical studies in Tribhuvan University, Central Campus, the only medical college existing in Nepal during that time. During her medical studies, she served as the treasurer of the Free Student's Union of the Institute of Medicine and performed one month of clinical clerkship in the Department of Surgery, Shalgremska Hospital, Gotenborg, Sweden. After completing the medical school training including one-year of compulsory rotating internship, she decided to start her research career abroad. From March 2000 to May 2000, she worked as a research assistant in the Department of Clinical Pharmacology, University of Groningen, the Netherlands. In September 2000, she joined PhD programme at the same place. After nearly a year of research in the field of Renin-Angiotensin System and Cardiac Fibrosis, she moved to Maastricht to continue her PhD with an affiliation to Cardiovascular Research Institute Maastricht, University of Maastricht. As an honour of her hard work, she was awarded several prizes and medals during her career as a student and research fellow.

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type I TGF β receptor prevents angiotensin II induced P38 activation, cardiac hypertrophy and dysfunction. *Submitted*

10. **Pokharel S**, de Boer RA, Sharma UC, van Leeuwen REW, Brigstock DR and Pinto YM. Stretch-induced connective tissue growth factor expression in cardiac fibroblasts is mediated by EGF-receptor-coupled β 1 integrin. *Submitted*
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Awards

1. Young investigator award: European Council for Blood Pressure and Cardiovascular Research, 2002 and 2003
2. Best poster award (Cardiac Biology): 8th Annual meeting of the European Council for Blood Pressure and Cardiovascular Research, 2003
3. Jamuna Devi Gold Medal (2000): Designated to the best performing medical student in Internal Medicine
4. Dr Laxmi Narayan Gold Medal (2000): Designated to the best performing medical student in Otolaryngology
5. Kabita Shrestha Gold Medal (2000): Designated to the overall best performing female medical student